# Delineation of a CpG Phosphorothioate Oligodeoxynucleotide for Activating Primate Immune Responses In Vitro and In Vivo[1]

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**Oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides within specific sequence contexts (CpG motifs) are detected, like bacterial or viral DNA, as a danger signal by the vertebrate immune system. CpG ODN synthesized with a nuclease-resistant phosphorothioate backbone have been shown to be potent Th1-directed adjuvants in mice, but these motifs have been relatively inactive on primate leukocytes in vitro. Moreover, in vitro assays that predict in vivo adjuvant activity for primates have not been reported. In the present study we tested a panel of CpG ODN for their in vitro and in vivo immune effects in mice and identified in vitro activation of B and NK cells as excellent predictors of in vivo adjuvant activity. Therefore, we tested >250 phosphorothioate ODN for their capacity to stimulate proliferation and CD86 expression of human B cells and to induce lytic activity and CD69 expression of human NK cells. These studies revealed that the sequence, number, and spacing of individual CpG motifs contribute to the immunostimulatory activity of a CpG phosphorothioate ODN. An ODN with a TpC dinucleotide at the 5′ end followed by three 6 mer CpG motifs (5′-GTCGTT-3′) separated by TpT dinucleotides consistently showed the highest activity for human, chimpanzee, and rhesus monkey leukocytes. Chimpanzees or monkeys vaccinated once against hepatitis B with this CpG ODN adjuvant developed 15 times higher anti-hepatitis B Ab titers than those receiving vaccine alone. In conclusion, we report an optimal human CpG motif for phosphorothioate ODN that is a candidate human vaccine adjuvant. *The Journal of Immunology*, 2000, 164: 1617–1624.**

Bacterial DNA, but not vertebrate DNA, has rapid immunostimulatory effects on leukocytes in vitro (**1**, 2). CpG dinucleotides are under-represented (CpG suppression, 1/50 to 1/60) and selectively methylated in vertebrate DNA, but are present at the expected frequency (1/16 bases) and unmethylated in bacterial DNA (3, 4). Teleologically, it appears likely that the recognition of unmethylated CpG dinucleotides within specific flanking bases (referred to as CpG motifs) may have evolved as an ancestral nonself pattern recognition mechanism used by the innate immune system to detect DNA of pathogens, such as bacteria and viruses (5). In mice the optimal CpG motif is an unmethylated CpG dinucleotide that is flanked by two 5′ purines and two 3′ pyrimidines, the best of which is 5′-GACGTT-3′ (6, 7). DNA containing these CpG motifs activates murine macrophages to secrete cytokines, especially IL-12, TNF-α, and IFN-αβ (8–13), and stimulates murine dendritic cells (14, 15) and murine B cells (16–18). Acting in synergy with the CpG DNA (which does not directly stimulate highly purified NK cells), the IL-12 secondarily activates murine NK cells to secrete IFN-γ (10, 13) and to have increased lytic activity (9). Overall, CpG DNA induces a predominantly Th1 pattern of immune activation.

Synthetic oligodeoxynucleotides (ODN)[3] containing the optimal murine CpG motif (5′-GACGTT-3′) are known to be excellent immune adjuvants in various murine disease models and to drive Th1 immune responses (19–25). They are comparable or superior to CFA (but without apparent toxicity) and are superior to the standard human adjuvant alum with respect to the induction of Ag-specific humoral and cell-mediated immune responses (19–22, 25). Murine CpG ODN induce potent anti-tumor immune activity (26) and induce resistance to lethal challenge with *L. monocytogenes* (27). Our studies also support the use of CpG DNA for the conversion of allergic Th2-type immune responses into nonallergic Th1 responses (28).

Recently, we found that phosphorothioate ODN with the purine-purine-CG-pyrimidine-pyrimidine formula that had been identified as the most stimulatory motif in mice show no or only weak activity in human immune cells (29). We identified a potent human CpG motif, 5′-GTCGTT-3′, by testing phosphodiester ODN for the ability to stimulate human primary B cells (29) and found that

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[3] Abbreviations used in this paper: ODN, oligodeoxynucleotides; HBsAg, hepatitis B surface Ag.



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a phosphodiester ODN with a single copy of the optimal human CpG motif triggers ~60% of human peripheral blood B cells to proliferate and express high levels of CD86. We also demonstrated that this ODN, of sequence 2080, promotes growth, activation, and maturation of human peripheral blood dendritic cells (30).

To have in vivo clinical utility, ODN must be administered in a form that protects them against nuclease degradation. The native phosphodiester internucleotide linkage can be modified to become highly nuclease resistant via replacement of one of the nonbridging oxygen atoms with a sulfur, which constitutes phosphorothioate ODN. However, a phosphorothioate backbone reduces the affinity of the CpG ODN to a putative CpG binding protein(s) (B. Noll, W. Shen, C. Schetter, M. Wold, and A. M. Krieg, manuscript in preparation). Of note, an ODN containing a single optimal human CpG motif followed by a poly C tail, which is highly active with a phosphodiester backbone, is essentially inactive with a phosphorothioate backbone. In contrast, murine leukocytes are strongly activated by phosphorothioate ODN containing just one optimal murine motif. This argues for differences in the precise mechanism of CpG recognition between human and murine immune cells.

The goal of the present study was to identify the sequence of a human CpG phosphorothioate ODN that would have optimal adjuvant activity in vivo. Because we saw not only quantitative but also qualitative differences in the activities of different CpG ODN in mice, we first screened a panel of CpG and non-CpG control ODN on mouse cells to find in vitro assays with reliable and strong correlation to in vivo adjuvant activity with hepatitis B surface Ag (HBsAg). We then systematically tested a panel of >250 phosphorothioate ODN in corresponding human assays to identify sequences with in vitro immunostimulatory activity. We next examined whether the ODN with the highest activity in these human assays also activate B cell proliferation in chimpanzees and monkeys, and finally, whether they are active as adjuvants with HBsAg in chimpanzees and cynomolgus monkeys in vivo.

## Materials and Methods

### *Oligodeoxynucleotides*

Phosphorothioate-modified ODN were purchased from Operon Technologies (Alameda, CA) and Hybridon Specialty Products (Milford, MA). The sequences used are provided in Table 1 and Fig. 1. ODN were tested for endotoxin using the LAL-assay (BioWhittaker, Walkersville, MD; lower detection limit, 0.1 endotoxin units /ml). For in vitro assays, ODN were diluted in TE buffer (10 mM Tris, pH 7.0, and 1 mM EDTA) and stored at −20°C. For in vivo use, ODN were diluted in PBS (0.1 M PBS, pH 7.3) and stored at 4°C. All dilutions were conducted using pyrogen-free reagents.

### *Mouse spleen cell cultures*

Spleens were removed from 6- to 12-wk-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME), $2 \times 10^6$ splenocytes were cultured with 0.2 $\mu$M ODN for 4 h (TNF-$\alpha$) or 24 h (IL-6, IFN-$\gamma$, IL-12), and cytokines were detected by ELISA as previously described (16). To evaluate CpG-induced B cell proliferation, spleen cells were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over Lympholyte M (Cedarlane Laboratories, Hornby, Canada), cultured for 44 h with the indicated ODN, and then pulsed for 4 h with 1 $\mu$Ci of [$^3$H]thymidine as described previously (5). To examine NK cell lytic activity, murine spleen cells were depleted of B cells using magnetic beads coated with goat anti-mouse Ig as previously detailed (31). Cells were cultured at $5 \times 10^6$/well in 24-well plates and harvested at 18 h for use as effector cells in a standard 4-h $^{51}$Cr release assay against YAC-1 target cells. One unit was defined as the number of cells needed to effect 30% specific lysis. ODN 1758 was also induced NK activity when tested at a higher concentration.

### *Immunization of mice against HBsAg and evaluation of the humoral response*

Groups of 6- to 8-wk-old female BALB/c mice ($n = 5$ or 10; Charles River, Montreal, Canada) were immunized against HBsAg as previously described (21). In brief, each mouse received a single i.m. injection of 50 $\mu$l of PBS containing 1 $\mu$g of recombinant HBsAg (Medix Biotech, Foster City, CA) and 10 $\mu$g of CpG ODN or non-CpG control ODN (see Table 1 for sequences) as a sole adjuvant or combined with alum (Alhydrogel "85," Superfos Biosector, Vedbaek, Denmark; 25 mg of $Al^{3+}$/mg of HBsAg). Control mice were immunized with HBsAg without adjuvant or with alum. Plasma was recovered from mice at various times after immunization, and Abs specific to HBsAg (anti-HBs) were quantified by end-point dilution ELISA (in triplicate) as described previously (21). End-point titers were defined as the highest plasma dilution that resulted in an absorbance value ($OD_{450}$) 2 times higher than that of nonimmune plasma with a cut-off value of 0.05.

### *Isolation of primate PBMC and cell culture*

PBMC were isolated from peripheral blood of healthy volunteers, chimpanzees, or rhesus or cynomolgus monkeys by Ficoll-Hypaque density gradient centrifugation (Histopaque-1077, Sigma, St. Louis, MO) as previously described (32). Cells were suspended in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT), 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (all from Life Technologies, Grand Island, NY; complete medium). Cells (final concentration, $1 \times 10^6$ cells/ml) were cultured in complete medium in a 5% $CO_2$ humidified incubator at 37°C. ODN and LPS (from *Salmonella typhimurium*; Sigma) or anti-IgM were used as stimuli. For measurement of human NK lytic activity, PBMC were incubated at $5 \times 10^6$/well in 24-well plates. Cultures were harvested after 24 h, and cells were used as effectors in a standard 4-h $^{51}$Cr release assay against K562 target cells as previously described (9, 31). For B cell proliferation, 1 $\mu$Ci of [$^3$H]thymidine was added 18 h before harvest, and the amount of [$^3$H]thymidine incorporation was determined by scintillation counting on day 5. The SDs of the triplicate wells were <5%.

### *Flow cytometry on primate PBMC*

Surface Ags on primate PBMC were stained as previously described (33). Monoclonal Abs to CD3 (UCHT1), CD14 (M5E2), CD19 (B43), CD56 (B159), CD69 (FN50), and CD86 (2331 (FUN-1)) were purchased from PharMingen (San Diego, CA). IgG1,$\kappa$ (MOPC-21) and IgG2b,$\kappa$ (30) were used to control for nonspecific staining. NK cells were identified by CD56 expression on CD3-, CD14-, and CD19-negative cells, whereas B cells were identified by expression of CD19. Flow cytometric data from 10,000 cells/sample were acquired on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). The viability of cells within the forward/side scatter gate used for analysis was examined by propidium iodide staining (2 $\mu$g/ml) and was > 98%. Data were analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Stanford, CA).

### *Immunization of chimpanzees and cynomolgus monkeys against HBsAg and evaluation of the humoral response*

Fourteen cynomolgus monkeys (2.0–3.5 kg) were immunized with a pediatric dose of Engerix-B (SmithKline Beecham Biologicals, Rixensart, Belgium) containing 10 $\mu$g of HBsAg adsorbed to alum (25 mg of $Al^{3+}$/mg of HBsAg). This was administered alone ($n = 5$) or combined with CpG ODN 1968 ($n = 5$; 500 $\mu$g) or CpG ODN 2006 ($n = 4$; 150 $\mu$g; see Fig. 2 for sequences). Four chimpanzees (10–20 kg) were immunized in the same fashion, with two receiving control vaccine (Engerix-B only) and two receiving experimental vaccine (Engerix-B plus 1 mg of CpG ODN 2006). All vaccines were administered i.m. in the right anterior thigh in a total volume of 1 ml. Monkeys were maintained in the animal facility of the Primate Research Center (Bogor, Indonesia), and chimpanzees were housed at Bioqual (Rockville, MD). Animals were monitored daily by animal care specialists. No symptoms of general ill health or local adverse reactions at the injection site were noted. Plasma was recovered by i.v. puncture before and at various times after immunization and was stored frozen (−20°C) until assayed for Abs. Anti-HBs Abs were detected using a commercial ELISA kit (Monolisa Anti-HBs, Sanofi-Pasteur, Montreal, Canada), and titers were expressed in milliinternational units per milliliter based on comparison with World Health Organization-defined standards (Monolisa Anti-HBs Standards, Sanofi-Pasteur).

## Results

### *Identification of CpG ODN with different profiles of in vitro immune activities*

Our previous studies showed that the precise bases on the 5′ and 3′ sides of a CpG dinucleotide within a CpG motif have a major impact on the level of immune activation of a synthetic ODN, but

Table I. *Correlation of in vitro and in vivo CpG ODN immunostimulatory effects*

| ODN | In Vitro[a] | | | | | | In Vivo[b] | |
|---|---|---|---|---|---|---|---|---|
| | NK activity (LU) | B cell (SI) | IL-12 (pg/ml) | IL-6 (pg/ml) | TNF-α (pg/ml) | IFN-γ (pg/ml) | Anti-HBs, no alum | Anti-HBs + alum |
| Media | 0 | 1 | 0 | 54 | 0 | 0 | 122 | 773 |
| 1982 (5′-TCCAGGACTTCTCTCAGGTT-3′) | 0 | 1.9 | 0 | 0 | 0 | 2 | 54 | 774 |
| 1983 (5′-TTTTTTTTTTTTTTTTTTTT-3′) | 0 | 1.2 | 183 | 223 | 0 | 28 | 66 | 638 |
| 1628 (5′-GGGGTCAACGTTGAGGGGGG-3′) | 0 | 6.0 | 417 | 377 | 0 | 303 | 501 | 2,480 |
| 1758 (5′-TCTCCCAGCGTGCGCCAT-3′) | 0 | 14.0 | 2,995 | 214 | 82 | 28 | 564 | 1,650 |
| 1760 (5′-ATAATCGACGTTCAAGCAAG-3′) | 2.0 | 25.7 | 3,612 | 631 | 302 | 144 | 1,372 | 3,574 |
| 1826 (5′-TCCATGACGTTCCTGACGTT-3′) | 4.9 | 23.7 | 6,777 | 6,343 | 513 | 478 | 3,887 | 28,360 |
| 1841 (5′-TCCATAGCGTTCCTAGCGTT-3′) | 4.0 | 25.5 | 3,926 | 2,026 | 279 | 64 | 3,760 | 18,400 |
| *r* vs anti-HBs (no alum) | 0.98 | 0.84 | 0.88 | 0.85 | 0.90 | 0.57 | | |
| *r* vs anti-HBs (with alum) | 0.95 | 0.70 | 0.86 | 0.91 | 0.88 | 0.68 | | |

[a] In vitro assays were carried out on spleen cells removed from BALB/c mice as described in *Materials and Methods*. Stimulation index was determined as the ratio of cpm in wells without ODN to that in wells that had been stimulated throughout the culture period with the indicated ODN. Each in vitro value is the mean of triplicate assays.

[b] In vivo assays were carried out by immunization of BALB/c mice with 1 μg HBsAg plus 10 μg of the indicated ODN, with or without alum (25 μg $Al^{3+}$). Mice were bled at 4 wk postimmunization, and plasma was assayed for IgG anti-HBs Abs by ELISA assay. Anti-HBs values are the mean of titers obtained from 10 individual animals, and these are themselves the mean of duplicate assays.

it has been unclear whether different CpG motifs might display different immune effects. To evaluate this possibility, we tested a panel of CpG ODN for the ability to induce NK lytic activity and B cell proliferation and to stimulate synthesis of TNF-α, IL-6, IFN-γ, and IL-12 in murine spleen cells (Table I). Immunostimulatory activity of ODN without CpG motifs (ODN 1982 and ODN 1983; Table I) was negative or weak compared with that of CpG ODN. Consistent with our earlier findings (5) ODN with nonoptimal CpG motifs (ODN 1628 and ODN 1758) were less active than ODN containing CpG motifs flanked by two 5′ purines and two 3′ pyrimidines (ODN 1760, ODN 1826, and ODN 1841). Within these ODN, ODN 1826 containing two optimal murine CpG motifs (5′-GACGTT-3′) had the highest activity for five of six measured end points. Except for ODN 1628, all ODN showed a generally similar pattern of activity (NK cell-mediated lysis, B cell proliferation, IL-12, IL-6, TNF-α, and IFN-γ). Of note, ODN 1628, which was unique in this panel for containing two G-rich regions, showed preferential induction of IFN-γ synthesis but relatively low stimulation of the other activities.

### *Identification of in vitro assays that correlate with in vivo adjuvant activity*

Because adjuvant activity is an in vivo end point, we were interested in identifying in vitro assays that would predict the adjuvant activity of a CpG ODN in vivo. The same ODN used for in vitro end points therefore were tested for their adjuvant activity to immunize mice against HBsAg. This was conducted both with ODN alone and with ODN combined with alum, since earlier studies had shown strong synergy for CpG ODN and alum adjuvants (21).

BALB/c mice immunized with HBsAg without adjuvant attained only low titers of anti-HBs by 4 wk, and this was not affected by addition of non-CpG control ODN. In contrast, addition of CpG ODN raised anti-HBs titers 5- to 40-fold depending on the sequence used (Table I). When alum was added, titers of anti-HBs were ~6 times higher than those with HBsAg alone. Nevertheless, the various ODN combined with alum gave similar levels of augmentation relative to alum alone, as was found with the nonalum formulations relative to no adjuvant. Specifically, non-CpG ODN had no effect, and the various CpG ODN augmented these titers 2- to 36-fold (Table I). Results obtained with the different ODN alone correlated very strongly ($r = 0.96$) with those obtained using the same ODN plus alum. When linear regression was performed, a very high degree of correlation was found between certain in vitro assays and in vivo augmentation of anti-HBs titers. Of all the in vitro end points examined, the induction of NK lytic activity showed the best correlation to in vivo adjuvant activity (without alum, $r = 0.98$; with alum, $r = 0.95$; $p < 0.0001$). A good correlation regarding adjuvant activity was also obtained for B cell stimulation ($r = 0.84$ and 0.7) as well as secretion of TNF-α ($r = 0.9$ and 0.88), IL-12 ($r = 0.88$ and 0.86), and IL-6 ($r = 0.85$ and 0.91; Table I). The one in vitro assay that did not correlate well with the in vivo results was IFN-γ secretion ($r = 0.57$ and 0.68; Table I). This was due to the preferential IFN-γ-inducing activity of ODN 1628, which alone among the ODN in this panel contained G-rich regions. These data demonstrate that in vitro assays for NKlytic activity, B cell activation, and production of TNF-α, IL-6, and IL-12 provide valuable information in vitro to predict the adjuvant activity of a given ODN in vivo.

### *Screening of a phosphorothioate ODN panel to activate human NK cells*

In previous studies we found that synthesis of inflammatory cytokines by human PBMC is induced by extremely low amounts of endotoxin (induced TNF-α secretion is detectable with just 6 pg/ml endotoxin, 2 logs more sensitive than murine immune cells) (34). In contrast, activation of human B cells and induction of human NK cell lytic activity with endotoxin are low even at high endotoxin concentrations. Based on these results we selected activation of NK cells (lytic activity and CD69 expression) and B cells (proliferation and CD86 expression) as the most highly specific and reproducible assays with low intersubject variability and used these assays for in vitro screening of a pool of ODN.

First we studied the effect of phosphorothioate ODN containing various combinations and permutations of CpG motifs on NK cell-mediated lysis of target cells. For clarity and ease of presentation, only data with selected representative CpG and control ODN are shown. Human PBMC were incubated with different phosphorothioate ODN (6 μg/ml) for 24 h and tested for their ability to lyse $^{51}$Cr-labeled K562 cells. ODN without CpG motifs (ODN 1982 and ODN 2010; Fig. 1), runs of CpGs, ODN with nonoptimal CpG motifs, ODN containing only one 6-mer CpG motif (either 5′-GACGTT-3′ or 5′-GTCGTT-3′ underlined for clarity), and ODN

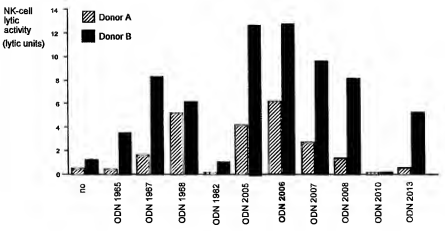


**FIGURE 1.** Induction of human NK cell lytic activity by phosphorothioate ODN. Human PBMC were incubated for 24 h with ODN (6 μg/ml) as indicated. Lysis of $^{51}$Cr-labeled K562 cells was determined, and lytic units were calculated as described in *Materials and Methods*. Data represent the mean of two independent experiments with two different donors.

containing two of these motifs without a TpC on the 5′ end of the ODN failed to induce NK lytic activity substantially above background. Examples of such nonactive ODN with CpG motifs are ODN 1781 (5′-ACCATGGACGTTCTGTTTCCCCTC-3′), ODN 1823 (5′-GCATGACGTTGAGCT-3′), and ODN 1829 (5′-ATGACGTTCCTGACGTT-3′; not shown in figure). ODN with two 6-mer CpG motifs (either 5′-GACGTT-3′ or 5′-GTCGTT-3′) in combination with a TpC at the 5′ end of the ODN (ODN 1840, 5′-TCCATGTCGTTCCTGTCGTT-3′; ODN 1851, 5′-TCCTGACGTTCCTGACGTT-3′; not shown in figure) or with at least three 6-mer motifs without a TpC at the 5′ end (ODN 2013; Fig. 2) show intermediate activity. High activity was found when the 5′ TpC directly preceded a 6-mer human CpG motif (5′-TCGTCGTT-3′) and was followed by two 6-mer motifs (ODN 2005, ODN 2006, and ODN 2007). The best results were obtained when the 6-mer CpG motifs were separated from each other and from the 5′ 8-mer CpG motif by TpT (ODN 2006).

Expression of the activation marker CD69 is rapidly up-regulated on the surface of NK cells subsequent to stimulation. To confirm the results from the NK cell lysis assay, PBMC were incubated for 18 h with ODN (2 μg/ml). CD69 expression was examined on CD56-positive NK cells (CD3, CD14, and CD19 negative). Although induction of CD69 expression was less sequence restricted than stimulation of NK cell functional activity, control ODN (ODN 1982, ODN 2116, ODN 2117, and ODN 2010) showed only low activity similar to background levels (Fig. 2). ODN with two human CpG motifs separated by 5′-TTTT-3′ (ODN 1965) or four human CpG motifs without spacing (ODN 2013) were relatively more active at inducing CD69 expression (Fig. 2, *left panel*) than at stimulating NK cell lytic activity (Fig. 1). Optimal NK cell functional activity as well as CD69 expression were obtained with ODNs containing a TpC dinucleotide preceding the human CpG motif and additional human motifs within the sequence (ODN 2006 and ODN 2007; Fig. 2, *left panel*).

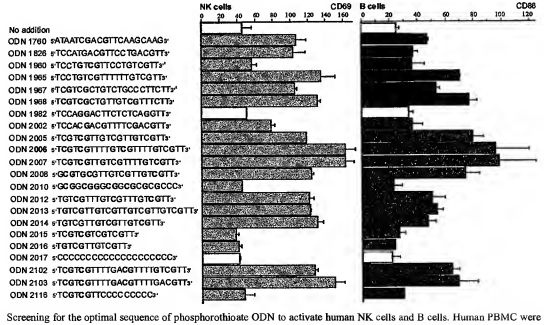


**FIGURE 2.** Screening for the optimal sequence of phosphorothioate ODN to activate human NK cells and B cells. Human PBMC were incubated with completely phosphorothioate-modified ODN (2 μg/ml for NK cells, *left panel*; 0.6 μg/ml for B cells, *right panel*) with the sequences indicated (CG dinucleotides in bold). CD69 expression was measured after 18 h on CD56-positive NK cells (negative for CD3, CD14, and CD19), and CD86 expression was measured after 48 h on CD19-positive B cells. The results show the means of experimental duplicates from two different donors for both NK cells and B cells.

*Activity of phosphorothioate ODN for stimulating human B cells*

In preliminary experiments we found that the percentage of proliferating B cells (5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) assay, see *Materials and Methods*) correlated with the surface expression of the costimulatory CD86 on B cells, as measured by flow cytometry. Thus, we used CD86 expression on B cells to screen a panel of ODN for immunostimulatory activity. PBMC were incubated with 0.6 μg/ml ODN. Expression of CD86 (mean fluorescence intensity) was examined on CD19-positive B cells (Fig. 2, *right panel*). A poly C ODN (ODN 2017) or ODN without CpG dinucleotides (ODN 1982) failed to stimulate human B cells under these experimental conditions. A phosphorothioate ODN (ODN 2116) with one optimal human CpG motif preceded by a TpC (5′-TCGTCGTT-3′) was inactive (Fig. 2, *right panel*). The presence of one human 6-mer CpG motif (5′-GTCGTT-3′) had no activating effect (not shown). Two of these CpG motifs within the sequence showed no (ODN 1960 and ODN 2016) or intermediate (ODN 1965) activity dependent on the sequence context. If the ODN was composed of three or four copies of this motif (ODN 2012, ODN 2013, and ODN 2014), intermediate activity on B cells could be detected. The combination of the human 8-mer CpG motif on the 5′ end of the ODN with two 6-mer CpG motifs (ODN 2005, ODN 2006, ODN 2007, ODN 2102, and ODN 2103) led to a considerable increase in the ability of the ODN to stimulate B cells. The spacing between the single motifs was critical. The separation of CpG motifs by TpT was preferable (ODN 2006) compared with that of unseparated CpG motifs (ODN 2005; also compare ODN 1965 to ODN 1960). The human 6-mer CpG motif (5′-GTCGTT-3′) was better than the optimal mouse 6-mer CpG motif (5′-GACGTT-3′) when combined with the human 8-mer CpG motif on the 5′ end (ODN 2006 vs. ODN 2102 and ODN 2103). A $(TCG)_{poly}$ ODN was inactive or only weakly active, as were ODN containing CpG dinucleotides flanked by guanines or other CpG dinucleotides (ODN 2010; Fig. 2). Taken together, the findings for NK cells and B cells showed consistently that of the ODN tested, ODN 2006 has the highest immunostimulatory activity on human immune cells.

*Comparative analysis of potency of CpG phosphorothioate ODNs in different primates*

Different CpG motifs are optimal to activate murine and human immune cells. Furthermore, the number and location of CpG motifs within an active phosphorothioate ODN are different in mice and humans. We were interested to know whether CpG phosphorothioate ODN show similar activity among different species of primates. We compared a panel of CpG ODN for their ability to induce B cell proliferation in humans, chimpanzees, and rhesus or cynomolgus monkeys. The capability of ODN to stimulate human B cell proliferation (Table II) correlated well with their ability to induce CD86 expression on B cells (Fig. 2, *right panel*). ODN 2006, which showed the highest activity in human B cells and NK cells, was also the most active in stimulating chimpanzee and rhesus monkey B cell proliferation (Table II). ODN 1968 and ODN 2006 gave the highest activation of cynomolgus monkey B cells in vitro (stimulation index, 25 and 29, respectively, at 6 μg ODN/ml). Surprisingly, CpG ODN 2007, which displayed similarly high activity as the optimal ODN 2006 in human cells, did not stimulate rhesus monkey or chimpanzee B cell proliferation, and ODN 1968 showed low activity. CpG ODN originally identified with high activity in mice (ODN 1760 and ODN 1826) showed little activity in monkeys (Table II).

Table II. *Proliferative response of PBMC to phosphorothioate CpG ODN in primates*[a]

| | Humans | Chimpanzee | Rhesus Monkey |
|---|---|---|---|
| No addition | 0.5 ± 0.1 | 0.5 ± 0.1 | 0.5 ± 0.0 |
| ODN 1760 | 23 ± 7 | 0.3 ± 0.1 | 0.5 ± 0.3 |
| ODN 1826 | 0.8 ± 0.1 | 0.4 ± 0.1 | 0.6 ± 0.1 |
| ODN 1968 | 35 ± 9 | 20.0 ± 3.8 | 1.9 ± 0.7 |
| ODN 1982 | 9.7 ± 1.1 | 2.5 ± 1.1 | 0.7 ± 0.1 |
| ODN 2006 | 58 ± 8 | 27.4 ± 8.9 | 6.3 ± 3.3 |
| ODN 2007 | 47 ± 11 | 0.5 ± 0.1 | 0.4 ± 0.2 |

[a] PBMC were prepared from peripheral blood and incubated with ODN (0.6 μg/ml) as indicated for 5 days. Proliferation was measured by uptake of [$^3$H]thymidine (cpm/1000) during the last 18 h. More than 95% of proliferating cells were B cells as determined using the 5- (and 6-) carboxy fluorescein diacetate succinimidyl (CFSE) assay. Four human probands, six chimpanzees, and two rhesus monkeys were tested. Standard error of means is indicated.

*In vivo adjuvant activity of CpG ODN in chimpanzees and cynomolgus monkeys*

To evaluate whether CpG ODN with strong in vitro stimulatory effects on primate cells had detectable adjuvant activity in vivo, cynomolgus monkeys and chimpanzees were immunized with Engerix B, which comprises HBsAg adsorbed to alum, alone or with added ODN 1968 (500 μg) or ODN 2006 (1 mg), respectively. The results in the cynomolgus monkeys and chimpanzees cannot be directly compared because different CpG ODN were used. Nevertheless, compared with controls not receiving CpG ODN, anti-HBs titers at 4 wk postprime and 2 wk postboost were 66- and 16-fold higher, respectively, in the monkeys, and 15- and 3-fold higher in the chimpanzees (Table III). Thus, a clear adjuvant effect of CpG ODN was seen, and this was particularly striking after a single immunization. Because the number of animals studied is small, the differences seen are qualitative rather than quantitative.

Table III. *Anti-HBs responses in primates immunized against HBsAg with CpG ODN*[a]

| Primate Species | *n* | CpG ODN | Anti-HBs (mIU/ml) 4 wk post-prime | Anti-HBs (mIU/ml) 2 wk post-boost |
|---|---|---|---|---|
| Cynomolgus monkey | 5 | None | 15 ± 44 | 4,880 ± 13,113 |
| | 5 | ODN 1968 (500 μg) | 995 ± 1,309 | 76,449 ± 42,094 |
| Chimpanzee | 2 | None | 6, 11 | 3,712, 4,706 |
| | 2 | ODN 2006 (1 mg) | 125, 135 | 9,640, 16,800 |

[a] Animals were immunized by i.m. injection of Engerix B containing 10 μg HBsAg adsorbed to alum, alone or with added CpG ODN. Cynomolgus monkeys were boosted at 10 wk and chimpanzees were boosted at 4 wk post-prime. Anti-HBs was determined by ELISA assay; values for monkeys are GMT ± SEM ($n$ = 5), whereas individual values for the two chimpanzees in each group are provided.

## Discussion

Previous studies have demonstrated that CpG ODN are superb vaccine adjuvants in mice (19–25). However, these mouse stimulatory ODN have shown more modest effects on human leukocytes (29). In this study we were interested in the identification of an optimal CpG ODN for use as a vaccine adjuvant in humans. In vivo screening of different CpG ODN for this purpose is not practical in primates, and for that matter, responses in nonhuman primates may not be predictive of in vivo effects in humans. Therefore, we wanted to identify an in vitro test that would predict in vivo efficacy. To do this, we evaluated several in vitro assays in mice for their predictive value of adjuvant activity in vivo in mice. CpG ODN-induced B cell and NK cell activation in murine spleen cells correlated particularly well with their ability to enhance the immune response against HBsAg. Based on the human CpG motif, 5′-GTCGTT-3′, previously identified using phosphodiester ODN, we designed a panel of CpG phosphorothioate ODN to identify the optimal sequence of a phosphorothioate ODN for activation of human B cells and NK cells. We found that the following characteristics contribute to the potency of a human CpG phosphorothioate ODN: 1) a TpC dinucleotide at the 5′ end followed by the human CpG motif (5′-GTCGTT-3′); 2) two additional human CpG motifs within the sequence; and 3) separation of adjacent CpG motifs by TpT. The ODN 2006, a 24 mer with three human CpG motifs, fulfills all three criteria and showed consistently the highest activity of all ODN tested. The ODN 2006 was an excellent adjuvant for the induction of an immune response in chimpanzees against HBsAg in vivo. In the presence of ODN 2006, anti-HBs titers at 4 wk after prime were more than 10-fold higher than levels considered to be protective (≥10 mIU/ml). In contrast, control chimpanzees receiving vaccine without ODN 2006 had anti-HBs near or below protective levels. In humans, the commercial vaccine, which contains alum as an adjuvant, is typically given in three doses. Although the majority of people respond well to two doses, the third dose ensures a very high (>90%) seroprotection rate.

The human stimulatory ODN 2006 shows weaker activity in mice compared with the highly active murine CpG ODN 1826 (our unpublished observations), supporting the concept of species specificity of CpG DNA recognition by immune cells. Although ODN 2006 was active in vitro in all primates tested, other CpG ODN, such as ODN 2007, had relatively high activity in human immune cells but no or a weaker effect in chimpanzees and rhesus monkeys. This argues for differences in the CpG recognition mechanism even within primates. Because bacterial DNA provides a large pool of CpG motifs, the evolutionary pressure to conserve the recognition mechanism for one single specific CpG motif is low. In contrast, the loss of binding of a transcription factor to its specific DNA motif would abolish its function. For the recognition of the presence of bacterial DNA, it, rather, makes evolutionary sense that different species develop their own optimized bacterial DNA recognition mechanism based on the bacterial environment to which they are exposed.

The good predictive value of B cell activation for in vivo vaccine adjuvant activity is most likely linked to the central role of B cells in the establishment of a specific immune response. Polyclonal proliferation of B cells (induced by CpG ODN) increases the likelihood of an Ag-specific B cell/Th cell match. Furthermore, enhanced expression of the costimulatory molecule CD86 on polyclonally expanded B cells activates Ag-specific Th cells. B cells also increase their CD40 expression in response to CpG ODN (29), improving the capability of CD40 ligand-expressing activated Th cells to stimulate B cells. Increased ICAM-1 synthesis on B cells facilitates the cell-to-cell contact (29). Thus, the activation status of polyclonal B cells plays a critical role during the initiation of a specific Ab response. The contribution of NK cell activity to the establishment of specific Abs is less obvious, and so the strong correlation between NK cell activation and in vivo adjuvant activity that was observed in Table I was unexpected. NK cells are part of the innate immune system and as such are involved in the first line of defense against pathogens. Most likely, the cytokine pattern produced by NK cells upon activation is closely related to the initiation of a specific immune response. Overall, IFN-γ secretion did not correlate well with in vivo adjuvant activity, but it remains possible that this may have contributed to the adjuvant activity of ODN 1628, especially because this ODN failed to induce any detectable NK lytic activity. The use of dendritic cell activation for the screening of CpG ODN may add valuable information in future studies and allow for the identification of CpG ODN other than or even more potent than ODN 2006.

As there is a 2-log higher endotoxin sensitivity of human than mouse primary monocytes, extreme caution is required to avoid endotoxin contamination of CpG DNA used for testing in the human system (34). Because TNF-α, IL-6, and IL-12 are produced by human monocytes in response to even low amounts of endotoxin, their value for high throughput in vitro screening assays is limited. On the other hand, human B cells and NK cells show only minor activation by endotoxin and thus are far more useful in testing for CpG DNA immunostimulatory activity.

Stimulation of cellular function in either NK or B cells (i.e., lytic activity, proliferation) requires a stronger CpG ODN than the induction of activation markers at their surface (CD69, CD86). For both cell types, the use of cell surface activation markers showed a higher nonspecific background, attributable to the phosphorothioate backbone, compared with the functional assays. This high sensitivity of surface markers requires the use of low ODN concentrations for optimal discrimination between ODN of similar activity. Thus, the use of surface markers allows the comparison of ODN with weak activity, while functional assays are preferred for comparing ODN with high activity. It is of note that the optimal ODN concentrations for stimulating B cells and NK cells differ. Although 0.6 μg/ml ODN is maximal to stimulate B cells, optimal NK cell activation may require 6 μg/ml ODN. Both B cell activation and NK cell functional activity are measured within freshly isolated PBMC. We found earlier that highly purified human primary B cells are activated by CpG DNA (29). The existence of a direct effect of CpG DNA on NK cells is less clear, and a secondary mechanism mediated by another cell type within PBMC might contribute to CpG-induced functional activity of NK cells.

It has been shown that some phosphorothioate ODN can induce human B cell proliferation (35, 36). Liang et al. found that the 6-mer 5′-TCGTCG-3′ at the 5′ end is critical for the activity of an ODN to stimulate human B cells at low concentrations, but that a $(TCG)_3$ ODN did not have higher activity. Poly T, A, C, or G ODN or ODN with a random sequence were not active in their assays. These results are in agreement with our findings. In addition, we demonstrate that the activity of an ODN is markedly enhanced if the 5′-TCGTCG-3′ is followed by TpT. This transforms the 5′-TCGTCG-3′ into a human CpG motif preceded by a TpC. This 8-mer motif followed by a poly C tail shows maximal activity if used as a phosphodiester ODN. but not as a phosphorothioate ODN (29). If a 5′ end 5′-TCGTCG-3′ is followed by another human CpG motif within the sequence, the activity is also increased. However, ODNs with three or four copies of the human CpG motif (5′-GTCGTT-3′) without the 5′-TCGTCGTT-3′ motif at the 5′ end showed only low activity. This is consistent with the study by Liang et al. (36), who tested ODNs with several copies of GACGTT, TGACGTT, or TGACGTC that were not particularly

potent. Consequently, combining the 5′-TCGTCGTT-3′ located at the 5′ end with additional 5′-GTCGTT-3′ motifs gave the best results in our study.

An important question is whether the immune effects of CpG motifs may be modified by the presence of other types of sequence motifs. To date, only two distinct immune effects of ODN sequences could be clearly identified: the effects due to CpG motifs and the effects due to G-rich motifs. We found a surprisingly poor correlation between ODN that induce strong IFN-γ secretion and those that are strong adjuvants. This poor correlation was largely due to the effects of ODN 1628, which was relatively weak as an adjuvant and at inducing secretion of most of the other cytokines, yet induced high level production of IFN-γ. A distinguishing feature of ODN 1628 is the presence of two G-rich regions, or poly G motifs, one of which has four Gs in a row, and the other of which has six Gs. Such poly G sequences show immunostimulatory effects that are distinct from CpG-mediated effects. For example, the level of IFN induction by a CpG ODN can be enhanced by poly G sequences at the ends of the same ODN (37). On the other hand, an ODN containing poly G sequences alone can block induction of IFN secretion by another ODN with a CpG motif (37). Poly G ODN can also block the production of IFN-γ induced by the mitogens Con A, bacterial DNA, or the combination of PMA and the calcium ionophore A23187 (44). This inhibition was only seen with the phosphorothioate backbone. Of note, poly G-rich ODN can also block the downstream effects of IFN-γ (38, 39). Also, we previously showed that the effects of poly G depend on the ODN backbone; poly G motifs increase the NK activity of chimeric ODN (phosphorothioate linkages on both ends, phosphodiester ODN in the middle), but reduce the NK activity of phosphorothioate ODN (9). Further studies will be required to determine whether the IFN-γ response to the poly G ODN 1628 is IL-12 independent and to identify the producing cell type.

Alum (e.g., $Al_2O_3$) was developed >75 yr ago and is still the only adjuvant approved for human use in most countries. Alum induces a Th2-type rather than a Th1-type immune response, appears to interfere with the development of cell-mediated immunity, and blocks activation of $CD8^+$ CTL (40). We showed earlier that CpG ODN induce a predominant IgG2a Ab response (Th1-like) to HBsAg in mice and, when the two adjuvants are used together, can even overcome the Th2 bias (IgG1) of alum for both Ab isotype and CTL responses (21). Furthermore, we found that the CpG ODN could induce HBsAg-specific CTL in young mice, in which a Th1 response normally is difficult to obtain (22, 41). Besides a shift toward a Th1 immune response, CpG ODN have the advantage over alum that it could be used as an adjuvant with live attenuated or multivalent vaccines that cannot be mixed with alum. In situations where it is necessary to overcome non- or hyporesponsiveness, the synergistic effect of CpG ODN and alum may be useful.

In preclinical studies antisense phosphorothioate ODN that are designed to inhibit target protein synthesis have been found to be safe at doses >100 mg/kg. In clinical antisense studies, phosphorothioate ODN have been used in doses up to 12 mg/kg with little drug-related toxicity (42, 43). This is ~30 times higher than the dose of 10 μg/mouse used in the present study (0.36 mg/kg). Phosphorothioate ODN can be produced on a large scale under good manufacturing practices conditions at a cost of approximately $200/g (42). Thus, the use of CpG ODN as adjuvant could significantly lower the cost of vaccination when repeated doses are normally required to induce a protective immunity (for example, with hepatitis B).

In conclusion, our study defines a CpG phosphorothioate ODN with high activity on human immune cells and with excellent adjuvant characteristics in chimpanzees. The design of this compound is based on the optimal human CpG motif and additional features that are essential if a phosphorothioate backbone is used. As a drug, ODN 2006 is inexpensive and easy to manufacture and is a candidate ODN for human clinical trials as an adjuvant for immunotherapy of cancer, infectious diseases, and allergy.

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# CpG DNA and LPS induce distinct patterns of activation in human monocytes

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A specific set of immune functions is switched on in response to DNA containing unmethylated CpG dinucleotides in particular base contexts ('CpG motifs'). Plasmid, viral vectors and antisense oligodeoxynucleotides used for DNA vaccination, gene replacement or gene blockade contain immunostimulatory CpG motifs which may have undesirable therapeutic activity. Although the immunostimulatory effects of CpG motifs on murine cells are well established, the evaluation of their possible effects on human cells is complicated by the higher LPS sensitivity of human leukocytes compared with those in mice. To address this issue, we analyzed CpG- and LPS-mediated immune activation of human PBMC. Thereby, the activity of LPS could be detected within 4 h using intracellular TNF staining of monocytes with flow cytometry at concentrations just one-thirtieth (0.0015 EU/ml) of the lowest detection limit for the routinely used LAL assay (0.05 EU/ml). In contrast to the rapid LPS response, CpG DNA-stimulated TNF and IL-6 synthesis in human monocytes was not detectable until 18 h. E. coli DNA induced IL-6 synthesis in a concentration-dependent manner (40 μg/ml E. coli DNA: 405 pg/ml ± 75 pg/ml; n = 7; IL-6 ELISA), but calf thymus DNA did not (<10 pg/ml). Likewise, the CpG oligodeoxynucleotides 1760 (phosphorothioate) and 2059 (diamidited) induced IL-6 synthesis, but the corresponding control oligonucleotides 1796 and 2077 did not. CpG DNA and LPS enhanced IL-6 synthesis synergistically. ICAM-1 expression on monocytes was increased 4.6-fold by E. coli DNA, 3.5-fold by 1760 and three-fold by 2059, compared with 1.8-fold by a maximal LPS stimulus and no change with non-CpG DNA. In conclusion, 'CpG-motifs' induce TNF, IL-6 and ICAM-1 expression in human monocytes, but the kinetics of this differ from that induced by LPS, which makes it possible to distinguish immune activation by these agents. These results have important implications for the clinical development of therapeutic DNA in humans.

Keywords: CpG; therapeutic DNA; gene therapy; DNA vaccination; oligodeoxynucleotide; endotoxin

## Introduction

Nucleic acids are an important tool for the development of genome-based therapeutic strategies. The sequencing of the human and other genomes enables the development of therapeutic DNA which broadens the spectrum of pharmacologic intervention in disease. In gene therapy, therapeutic DNA is designed to replace defective genes or to provide new genes. Vaccination with naked DNA induces antigen-specific immune responses.[1] Antisense oligodeoxynucleotides (ODN) block protein synthesis by sequence-specific hybridization to the corresponding mRNA.[2–4] All these concepts are based on the view of DNA as a blueprint of proteins. However, DNA is more than that. Recently it has been discovered that unmethylated CpG dinucleotides in particular base contexts ('CpG-motifs') are recognized by the immune system as a danger signal.[5–8] Recognition as danger by the immune system is based on under-representation and methylation of CpG dinucleotides in vertebrate DNA compared with bacterial DNA. Synthetic DNA and DNA generated in bacterial systems contain unmethylated CpG motifs which stimulate the immune system. This has clear implications for DNA-based therapeutic strategies. Immune activation by CpG motifs in DNA used for gene therapy is unwanted since activated immune cells recognize and eliminate transfected cells expressing newly acquired antigens. In contrast, concomitant CpG-mediated immune stimulation is required in naked DNA vaccination for the stimulation of a specific immune response.[9,10] CpG motifs in antisense ODN can alter biologic functions in addition to the antisense mechanism itself.[11,12] Depending on the target protein and the target disease, this can be beneficial or unwanted.

In animal models, ODN with immunostimulatory CpG motifs have been shown to be of therapeutic value as adjuvants for conventional and therapeutic vaccination against infectious disease[13,14] and tumors.[15,16] CpG DNA biases the immune response to a Th1-like pattern (IL-12, IFN-γ, $IgG_{2a}$).[14,17,18] This shift is useful for the treatment of allergic disease[19] and to prevent or overcome infections with parasites or intracellular bacteria.[20,21] In mice, the adjuvant effect of CpG DNA is equally as potent as the complete Freund's adjuvant.[15,18] Complete Freund's adjuvant consists of heat-killed dried *Mycobacterium tuberculosis* which includes mycobacterial DNA and components of the cell membrane including endotoxins.[22–24] Bacterial DNA has been known for its immunostimulatory activity for many years,[25,26] but the CpG-mediated mechanism

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responsible for this activity was unknown until recently.[5] Endotoxins in complete Freund's adjuvant contribute to its toxicity[27] which hamper its therapeutic use in humans in contrast to mice which are less sensitive to LPS.

CpG effects on the immune system are well characterized in mice. CpG stimulates murine macrophages to release IL-12 and tumor necrosis factor (TNF),[28,29] two key cytokines in immune regulation.[30,31] Murine NK cells produce IFN-γ in response to CpG-induced TNF and IL-12 production by macrophages.[32–34] IFN-γ in turn costimulates macrophages for enhanced TNF and IL-12 synthesis.[29] Murine B-lymphocytes are directly activated by CpG to proliferate and to secrete Ig and IL-6.[5,35–37] In contrast, in humans CpG-mediated induction of cytokines[9,34,38,39] and induction of B cell proliferation[40–42] is less well characterized. Two major differences between mouse and human hinder the direct transfer of experimental settings from mice to humans: (1) the optimal flanking bases of the CpG dinucleotide are different in humans (A Krieg, unpublished data); and (2) human immune cells respond to 2 logs lower endotoxin levels than mouse cells. To evaluate immune effects of CpG DNA in humans we pursued the following strategy *in vitro*: (1) we examined *E. coli* DNA, which contains a variety of immunostimulatory CpG motifs, to estimate the capability of human immune cells to respond to CpG; (2) we compared ODN with CpG motifs to *E. coli* DNA; and (3) we analyzed the contribution of CpG and LPS to immune activation, using a newly developed, highly sensitive assay for LPS.

In the present study we show that: (1) CpG DNA elicits immune responses in human immune cells; (2) CpG- and LPS-mediated immune response can be distinguished by the time course of cytokine induction; and (3) CpG DNA and LPS act synergistically to induce inflammatory cytokines.

## Results

### *Detection of small amounts of endotoxin in DNA samples*

In previous studies, we and others have reported that CpG motifs in DNA induce synthesis of the pro-inflammatory cytokines TNF and IL-6 by murine spleen cells.[36] The current studies were performed to investigate the cytokine production by different cell types in human PBMC in response to *E. coli* DNA (containing CpG motifs), calf thymus DNA (nonstimulatory DNA) and LPS as a positive control. To perform the analysis we used intracellular cytokine staining which allows the detection of cytokine synthesis on the single cell level.

Freshly prepared human PBMC were incubated with these stimuli for 4 h in the presence of brefeldin A (1 μg/ml) to block cytokine secretion (see Materials and methods section). Detection of LPS by this method was highly sensitive. In PBMC from three different donors, the maximal dose of LPS (10 ng/ml) induced an average of 96.0% ± 1.2% monocytes (range, 94.2 to 97.6%, $n = 3$) to stain positive for TNF (one typical donor shown in Figure 1, first row, first plot: 94%). We found a dose-dependent decrease in TNF-positive monocytes for lower concentrations of LPS. The lower detection limit of this assay was 0.006 ng/ml LPS (Figure 1, third row, first plot: 3.7% compared with 0.9% without LPS).

In PBMC stimulated by 30 μg/ml *E. coli* DNA, 93% of monocytes stained positive for TNF (Figure 1, third row, second plot), equally effective to 10 ng/ml LPS (Figure 1, first row, first plot). However, there was a high background level of 34% TNF-positive cells induced by 30 μg/ml calf thymus DNA which was expected to be nonstimulatory based on our previous studies with murine cells (Figure 1, third row, third plot). Since human monocytes are far more sensitive to endotoxin than murine cells, we speculated that low levels of endotoxin contamination may be responsible for this TNF induction. In the LAL assay, the *E. coli* DNA sample contained 1.01 EU/ml, the calf thymus DNA sample 0.14 EU/ml endotoxin (final DNA concentration 30 μg/ml). Surprisingly, by careful purification of both DNA samples (see Materials and methods section), TNF induction disappeared completely for both *E. coli* DNA and calf thymus DNA (Figure 1, third row, second and third plot, A: unpurified DNA, B: purified DNA; compare with control without LPS: 0.9% TNF positive cells). *Importantly, even DNA samples already negative in the* LAL assay (<0.03 EU/ml) induced considerable TNF staining of monocytes, showing that the LAL assay is less sensitive compared with endotoxin detection by TNF staining with flow cytometry. Purified *E. coli* DNA and ODNs were also found to be negative for induction of TNF expression in human PBMC during the first 4 h (Figure 1, fourth row, second and third plot).

### *CpG DNA-induced TNF and IL-6 synthesis is delayed compared with LPS*

These results suggested a lack of TNF synthesis in human PBMC in response to endotoxin-free *E. coli* DNA or CpG ODN (LPS below 0.006 ng/ml). However, in these experiments TNF synthesis was measured only during the first 4 h. Adding brefeldin A (blockade of protein secretion) during later time spans allows the detection of TNF synthesis during later time periods. Between 4 and 10 h, the percentage of TNF-positive monocytes induced by 10 ng/ml LPS was still high (98%, Figure 2a, first row, first plot). Between 18 and 24 h, TNF-positive monocytes could no longer be detected (Figure 2a, second row, first plot) compared with the control without LPS (Figure 2a, second row, third plot). Furthermore, in the presence of lower LPS concentrations (1 ng/ml to 0.013 ng/ml) TNF-positive monocytes could not be detected either between 18 and 24 h (data not shown). Surprisingly, the purified *E. coli* DNA (30 μg/ml) stimulated TNF synthesis in 19% of monocytes between 18 and 24 h (Figure 2a, second row, second plot). No induction of TNF expression by *E. coli* DNA was found earlier between 4 and 10 h (Figure 2a, first row, second plot). The results of three independent experiments confirmed that *E. coli* DNA-induced TNF synthesis is delayed compared with LPS-induced TNF synthesis.

TNF and IL-6 are both pro-inflammatory cytokines produced by monocytes. Using the same technique as for TNF, we found an average of 95.8% ± 1.1% IL-6 positive monocytes (range 94.3 to 98.0, $n = 3$) during the first 4 h after stimulation with 10 ng/ml LPS. Between 4 and 10 h, 57% (Figure 2b, first row, first plot), and between 18 and 24 h 45% (Figure 2b, second row, first plot) of LPS-stimulated monocytes were IL-6 positive despite having become TNF-negative. Therefore, compared with the rapid decrease of LPS-induced TNF, LPS-induced IL-6

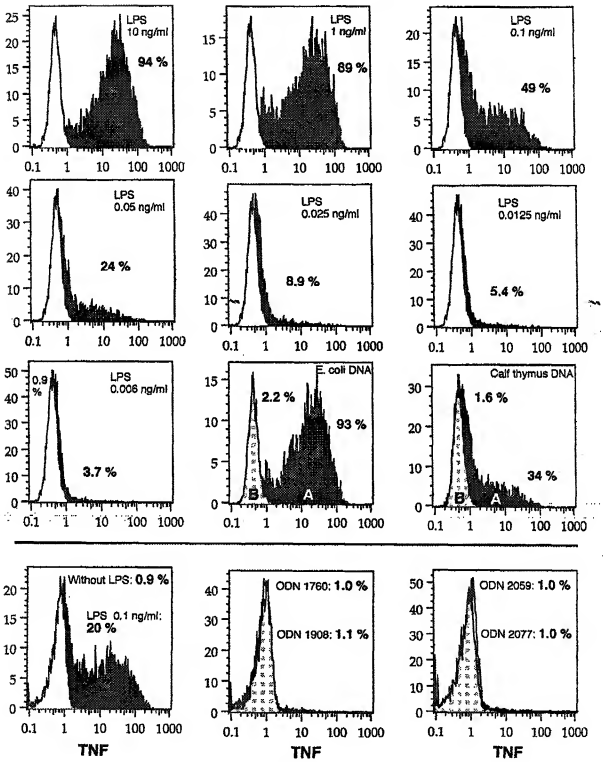


*Figure 1 Detection of small amounts of endotoxin in DNA samples by identification of TNF positive monocytes in flow cytometry. To detect endotoxin contamination in DNA samples, the high sensitivity of human monocytes to endotoxin was used. This assay has a 20-fold higher sensitivity than the LAL assay. Human PBMC ($1 \times 10^6$ cells/ml) were incubated for 4 h with either LPS (10 ng/ml to 0.006 ng/ml) or DNA samples. Protein secretion was blocked with brefeldin A (1 μg/ml). Monocytes were identified by CD14. After intracellular staining of cells for TNF, the percentage of TNF-positive monocytes was examined. LPS concentrations down to 0.006 ng/ml increased the percentage of TNF-positive monocytes compared with the control without LPS. Unpurified (a) E. coli DNA was found to be highly contaminated with endotoxin. After intense purification endotoxin contamination was below 0.006 ng/ml (b). Purification of calf thymus DNA was documented in the same way. Purified ODN (ODN 1760, 1908, 2059 and 2077) were found to be endotoxin-negative (separate experiment, lower panel).*

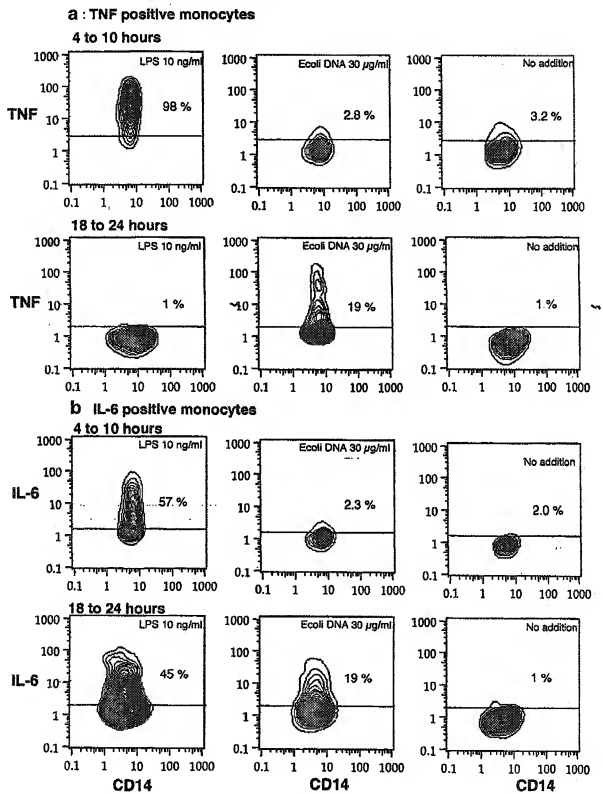


*Figure 2 CpG DNA-mediated induction of TNF and IL-6 in monocytes is delayed compared with LPS. PBMC ($1 \times 10^6$ cells/ml) were incubated in the presence of 10 ng/ml LPS (first column), 30 µg/ml E. coli DNA (second column) and without stimulus (third column). Brefeldin A (1 µg/ml) was added at 4 h or 18 h to block cytokine secretion. TNF (a) and IL-6 (b) synthesis from 4 to 10 h and from 18 to 24 h were determined in CD14-positive monocytes by intracellular staining of TNF and IL-6 and detection of positive cells by flow cytometry. E. coli DNA induced TNF and IL-6 synthesis between 18 and 24 h but not during earlier time spans. At the time LPS-induced IL-6 synthesis is still sustained (18 to 24 h) LPS-induced TNF synthesis already disappeared.*

showed a sustained production up to at least 24 h. *E. coli* DNA (30 μg/ml) stimulated 19% of the monocytes to produce IL-6 between 18 and 24 h (Figure 2b, second row, second plot). Similar to *E. coli* DNA-induced TNF, *E. coli* DNA-induced IL-6 production was not enhanced at earlier time-points.

Neither *E. coli* DNA nor LPS induced detectable TNF and IL-6 expression in B-lymphocytes or T-lymphocytes. In contrast, a combination of the stimuli phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) and calcium ionophore A23187 (250 ng/ml) as a positive control induced 14.6% of the B-lymphocytes and 7.1% of the T-lymphocytes to stain positive for TNF demonstrating that the assay has sufficient sensitivity to detect this (data not shown). Therefore the source of CpG DNA-induced TNF and IL-6 production in human PBMC appears to be monocytes. However, monocyte-restricted TNF and IL-6 production does not exclude the involvement of other cell types in the CpG response.

### *IL-6 production in response to CpG DNA is concentration dependent and CpG-specific*

Using intracellular cytokine staining as described above, it became clear that TNF and IL-6 synthesis in PBMC during the first 4 h after stimulation was induced by endotoxin. In contrast, analysis of highly purified DNA samples revealed that CpG DNA-mediated TNF and IL-6 expression was delayed compared with that induced by endotoxin. These purified DNA samples were used to stimulate PBMC for 24 h. To confirm that the IL-6 produced was secreted, IL-6 production was measured in the supernatant by ELISA (lower detection limit 10 pg/ml). All reagents used for cell culture were essentially endotoxin-free since cells incubated for 24 h without stimulus produced no detectable amounts of IL-6 (<10 pg/ml). *E. coli* DNA-induced IL-6 production was found to be CpG-specific and concentration dependent (Figures 3 and 4). In seven independent experiments, *E. coli* DNA (30 μg/ml) induced the production of an average of 409 pg/ml IL-6 (s.e.m. ± 75, range 182 to 670 pg/ml, $n = 7$) (Figure 3). In comparison, 0.1 ng/ml LPS induced the production of an average of 1283 pg/ml IL-6 (s.e.m. ± 355, range 475 to 3136 pg/ml, $n = 7$). Calf thymus DNA

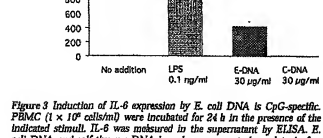


*Figure 3 Induction of IL-6 expression by E. coli DNA is CpG-specific. PBMC (1 × 10⁶ cells/ml) were incubated for 24 h in the presence of the indicated stimuli. IL-6 was measured in the supernatant by ELISA. E. coli DNA and calf thymus DNA have been proven to be endotoxin-free in flow cytometry. E. coli DNA-induced IL-6 (30 μg/ml) is compared with LPS (0.1 ng/ml). CpG specificity of E. coli DNA-induced IL-6 production is controlled by calf thymus DNA (30 μg/ml). Results are shown as means of seven independent experiments ± s.e.m.*

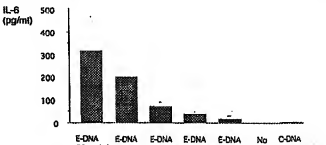


*Figure 4 Induction of IL-6 by E. coli DNA is concentration-dependent. PBMC (1 × 10⁶ cells/ml) were incubated in the presence of different concentrations of E. coli DNA for 24 h. IL-6 was measured in the supernatant by ELISA. E. coli DNA and calf thymus DNA were endotoxin-free as tested in flow cytometry. Results are given as means of two independent experiments.*

(30 μg/ml) induced no IL-6 synthesis (<10 pg/ml). In addition to calf thymus DNA, chloroquine was used to control for CpG specificity since it has been reported to block CpG-but not LPS-mediated cytokine expression selectively.[39,43] Chloroquine blocked *E. coli* DNA-induced IL-6 expression by 58% (52 to 65%, $n = 2$, not shown in Figure). In contrast, LPS-induced IL-6 was only slightly reduced by chloroquine (9%, 5 to 14%, $n = 2$). *E. coli* DNA induced IL-6 expression in a concentration-dependent manner. Lowering the concentration of *E. coli* DNA from 30 μg/ml to 10 μg/ml reduced IL-6 levels from 315 pg/ml to 18 pg/ml (Figure 4).

### *CpG DNA and LPS synergize for induction of IL-6 expression*

Since we found that CpG DNA and LPS show a different time-course of inducing cytokine expression, it is possible that CpG DNA and LPS use different pathways for their stimulatory activity. Therefore we examined if CpG DNA and LPS induce cytokine expression synergistically by adding 30 μg/ml *E. coli* DNA to increasing concentrations of LPS (Figure 5). The lowest LPS concentration which induced detectable IL-6 expression was 0.025 ng/ml (185 pg/ml, 53 to 316 pg/ml, $n = 2$). *E. coli* DNA alone induced 364 pg/ml IL-6 (324 to 404 pg/ml, $n$

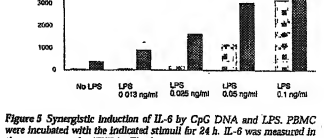


*Figure 5 Synergistic induction of IL-6 by CpG DNA and LPS. PBMC were incubated with the indicated stimuli for 24 h. IL-6 was measured in the supernatant by ELISA. The lower detection limit of the ELISA is 10 pg/ml. Endotoxin contamination of E. coli DNA was below 0.006 ng/ml as tested by TNF induction measured by flow cytometry. Results are given as means of two independent experiments. Error bars indicate the range.*

= 2) The addition of 30 μg/ml *E. coli* DNA to 0.025 ng/ml LPS increased IL-6 production 3.5-fold compared with the sum of IL-6 induced by LPS and *E. coli* DNA alone (Figure 5). This synergistic effect was found for all tested LPS concentrations, but was more pronounced for lower LPS concentrations. Even the lowest tested LPS concentration, 0.0125 ng/ml, which itself did not induce detectable amounts of IL-6, enhanced *E. coli* DNA-mediated IL-6 synthesis by 2.6-fold (from 364 pg/ml for *E. coli* DNA alone to 935 pg/ml, range 620 to 1250 pg/ml, $n = 2$). This synergistic effect was CpG-specific, since calf thymus DNA (30 μg/ml) did not influence LPS-stimulated IL-6 synthesis (tested for LPS concentrations 0.003 to 0.1 ng/ml, not shown).

### *CpG DNA induces strong ICAM-1 expression in monocytes*

The adhesion molecule ICAM-1 is an activation marker of monocytes and regulates intercellular communication between leukocytes. To investigate the possible effects of CpG DNA on ICAM-1 expression, human PBMC were incubated with *E. coli* DNA or LPS, and ICAM-1 expression was examined on CD14-positive monocytes by flow cytometry. We found that ICAM-1 is strongly upregulated in the presence of CpG DNA or LPS in a time-dependent manner. ICAM-1 expression increased continuously for both *E. coli* DNA and LPS until 48 h of stimulation (latest time-point tested). As soon as 6 h after stimulation ICAM-1 expression was enhanced for both *E. coli* DNA and LPS. Compared with *E. coli* DNA, LPS-induced ICAM-1 expression was higher at 6 h and at 24 h. However, at 48 h, *E. coli* DNA-induced ICAM-1 expression was even higher than LPS-induced ICAM-1. In all of the following experiments we measured ICAM-1 expression 48 h after stimulation.

Maximal ICAM-1 expression was induced by 0.1 ng/ml LPS with no further increase for higher doses of LPS. In the presence of 1 ng/ml LPS, the mean fluorescence intensity (MFI) of ICAM-1 staining was increased 3.6-fold (50.6 ± 6.3, 35.7 to 66.3, $n = 5$, $P = 0.001$) compared with the control without stimulus (13.9 ± 1.9, 10.3 to 20.6, $n = 5$) (Figure 6). *E. coli* DNA seemed to induce even higher expression of ICAM-1 (MFI: 63.3 ± 8.3, range 41.1 to 85.8, $n = 5$, $P < 0.001$), but the difference from 1 ng/ml LPS was not statistically significant ($P = 0.2$). A total of five experiments demonstrated that the induction of ICAM-1 expression by *E. coli* DNA (30 μg/ml) was not significantly different from ICAM-1 induction by 1 ng/ml LPS. The effect of *E. coli* DNA was CpG-specific, since ICAM-1 expression by calf thymus DNA was lower (MFI: 25 ± 3.9, 17.7 to 36.6, $n = 5$) although still enhanced compared with the control without stimulus (13.9 ± 1.9, 10.3 to 20.6, $n = 5$).

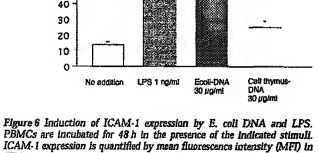


*Figure 6 Induction of ICAM-1 expression by E. coli DNA and LPS. PBMCs are incubated for 48 h in the presence of the indicated stimuli. ICAM-1 expression is quantified by mean fluorescence intensity (MFI) in CD14-positive monocytes using flow cytometry. Results are depicted as means of five independent experiments. Statistical evaluation is performed by the unpaired t test, error bars indicate s.e.m.*

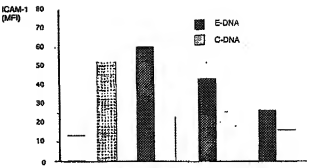


*Figure 7 Concentration-dependent induction of ICAM-1 in monocytes by E. coli DNA. PBMCs were incubated in the presence of different concentrations of E. coli DNA, calf thymus DNA and LPS for 48 h. ICAM-1 expression was quantified by flow cytometry in CD14-positive monocytes. Results are shown as means of two independent experiments. Error bars indicate the range.*

### *No synergistic effect of CpG DNA and LPS on ICAM-1 induction*

*E. coli* DNA and LPS are approximately equally effective in enhancing ICAM-1 expression in monocytes. However, in contrast to IL-6 induction, the addition of increasing concentrations of LPS to *E. coli* DNA (30 μg/ml) did not further increase ICAM-1 expression by *E. coli* DNA alone (Figure 8). The addition of calf thymus DNA did

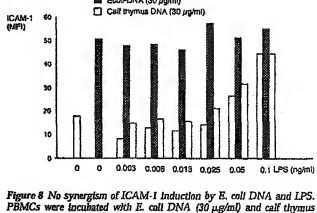


*Figure 8 No synergism of ICAM-1 induction by E. coli DNA and LPS. PBMCs were incubated with E. coli DNA (30 μg/ml) and calf thymus DNA (30 μg/ml) alone or in combination with different concentrations of LPS for 48 h. ICAM-1 expression was measured by flow cytometry in CD14-positive monocytes.*

not change the dose-dependent increase of LPS-mediated ICAM-1 induction, adding further evidence for the CpG specificity of *E. coli* DNA-induced ICAM-1 induction (Figure 8). To exclude that 30 μg/ml *E. coli* DNA alone induces the maximum amount of ICAM-1 expression, we tested a submaximal dose of *E. coli* DNA (7.5 μg/ml). Again, the addition of LPS did not increase the effect of 7.5 μg/ml *E. coli* DNA, although ICAM-1 expression by 7.5 μg/ml *E. coli* DNA was not maximal (MFI 58.0) compared with ICAM-1 induction by 30 μg/ml *E. coli* DNA (MFI 85.8) (not shown in Figure).

### *Induction of IL-6 and ICAM-1 expression is CpG-specific as tested by ODN with CpG motifs*

We hypothesized that the specific induction of IL-6 and ICAM-1 expression by *E. coli* DNA compared with calf thymus DNA may be due to their different content of unmethylated CpG motifs, which have been reported to account for the immunostimulatory effects of *E. coli* DNA on murine cells.[30] To prove CpG specificity as opposed to some other property of *E. coli* DNA, we tested two ODN with CpG motifs and their corresponding non-CpG control ODN for their capability to induce IL-6 and ICAM-1 expression in a CpG-dependent manner. The ODN 1760 is a nuclease resistant completely phosphorothioate-modified 20-mer and ODN 1908 is the corresponding control. The ODN 2059 (24-mer) is unmodified and ODN 2077 is the corresponding control. These purified ODNs were found to be endotoxin-free in flow cytometry (endotoxin lower than 0.006 ng/ml) (Figure 1, fourth row, second and third plot).

Because of their higher stability, the phosphorothioate-modified ODN (1760 and 1908) were used in a lower concentration (6 μg/ml) than the unmodified ODN (2059 and 2077, 30 μg/ml). Furthermore, the unmodified ODN were added repeatedly (0, 4 and 8 h). Both CpG ODN induced IL-6 expression after 24 h of incubation (1760: 93 pg/ml, 87 to 96 pg/ml; 2059: 60 pg/ml, 58 to 61 pg/ml, $n = 2$) (Figure 9). No IL-6 expression was induced by the control ODN (below 10 pg/ml). For the unmodified ODN 2059, the repeated addition was necessary. A single addition of 30 μg/ml 2059 induced no detectable IL-6 expression. A second addition showed lower IL-6 expression than three doses of the oligonucleotide.

Both CpG ODNs induced ICAM-1 expression after 48 h of incubation. ICAM-1 induction quantified by mean fluorescence intensity (MFI) was CpG-specific and in the same range as LPS-induced ICAM-1 synthesis (1760: 49, 47 to 51; 2059: 41, 40 to 42, $n = 2$) (Figure 10). Both control ODNs showed slightly enhanced ICAM-1 expression (1908: 28, 25 to 31; 2059: 25, 22 to 29, $n = 2$) which was comparable to ICAM-1 expression in the presence of calf thymus DNA (25 ± 3.9, 17.7 to 36.6, $n = 5$, Figure 6).

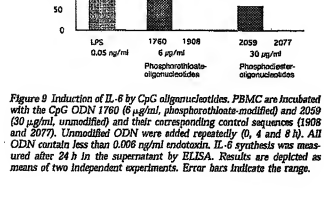


*Figure 9 Induction of IL-6 by CpG oligonucleotides. PBMC are incubated with the CpG ODN 1760 (6 μg/ml, phosphorothioate-modified) and 2059 (30 μg/ml, unmodified) and their corresponding control sequences (1908 and 2077). Unmodified ODN were added repeatedly (0, 4 and 8 h). All ODN contain less than 0.006 ng/ml endotoxin. IL-6 synthesis was measured after 24 h in the supernatant by ELISA. Results are depicted as means of two independent experiments. Error bars indicate the range.*

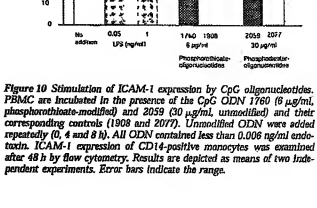


*Figure 10 Stimulation of ICAM-1 expression by CpG oligonucleotides. PBMC are incubated in the presence of the CpG ODN 1760 (6 μg/ml, phosphorothioate-modified) and 2059 (30 μg/ml, unmodified) and their corresponding controls (1908 and 2077). Unmodified ODN were added repeatedly (0, 4 and 8 h). All ODN contained less than 0.006 ng/ml endotoxin. ICAM-1 expression of CD14-positive monocytes was examined after 48 h by flow cytometry. Results are depicted as means of two independent experiments. Error bars indicate the range.*

## Discussion

CpG-mediated immune activation in mice is well characterized but information about CpG effects on human immune cells is far more limited. A better understanding of CpG effects in humans is required to transfer knowledge acquired from mouse studies to human therapeutic strategies.

Because human PBMC respond to 2 logs lower endotoxin levels than murine macrophages, we were not able to distinguish between CpG DNA- and LPS-mediated effects in the presence of even minor contamination of the DNA sample with LPS. After intense purification of *E. coli* DNA, a different time course of CpG- and LPS-mediated cytokine production was found using flow cytometry to measure cytokine synthesis by individual monocytes. LPS induced a rapid TNF and IL-6 synthesis during the first 4 h after stimulation. After 18 h no LPS-mediated TNF synthesis could be detected in the monocytes, while IL-6 production was still elevated. In contrast, the cytokine response to *E. coli* DNA was delayed and could not be detected earlier than 10 h after stimulation. The rapid TNF response to LPS during the first 4 h makes it possible to detect small amounts of LPS even in the presence of CpG motifs, because CpG-stimulated TNF appeared at later time-points. Detection of TNF-positive monocytes by flow cytometry allowed us to

detect as low as 0.006 ng/ml (0.0014 EU/ml) LPS. This technique was used to monitor proper purification of DNA samples down to a purification degree where non-CpG-mediated stimulation of monocytes was excluded.

With purified DNA samples we found potent and CpG-specific immune effects of both *E. coli* DNA and ODN with CpG motifs. *E. coli* DNA and CpG ODN induced expression of the proinflammatory cytokines TNF and IL-6 and the adhesion molecule ICAM-1 in human monocytes. IL-6 and TNF production could not be detected in PBMC cell types other than monocytes. While CpG-induced IL-6 expression was consistent but lower than LPS-stimulated IL-6, ICAM-1 induction by CpG DNA was even higher than LPS-induced ICAM-1 expression. Furthermore, CpG ODN induced ICAM-1 expression in the same range as *E. coli* DNA. In contrast, *E. coli* DNA stimulated approximately four times higher IL-6 production than CpG ODN.

CpG-mediated upregulation of ICAM-1 on leukocytes has not been described to date. The adhesion molecule ICAM-1 is a key mediator for the interaction of immune cells. Blockade of ICAM-1 by specific antibodies inhibits the mixed lymphocyte reaction.[44] Specific downregulation of ICAM-1 by antisense ODN improves Crohn's disease.[45] ICAM-1-mediated cell contact is required for the interaction between T-lymphocytes and antigen-presenting cells[46] and for transepithelial migration of immune cells.[47] ICAM-1, acting as a costimulatory molecule, is required but not sufficient for many inflammatory responses suggesting that its increased expression in response to CpG may be important in mediating the biologic effects of CpG DNA.

We found a synergism of CpG and LPS for induction of IL-6 synthesis. This synergism might be involved in human disease. In sepsis, bacterial DNA present in the circulation could trigger excessive cytokine production and increase the risk of a fatal outcome of disease. Even with the lower LPS sensitivity in mice, CpG and LPS were found to synergize for the production of TNF *in vivo*.[48] Recently it was shown that CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract suggesting that CpG DNA may play an important pathogenic role in inflammatory lung disease.[49] In chronic inflammatory bowel disease, the loss of the mucosal integrity could expose immune cells to both LPS and bacterial DNA present at high levels in the lumen of the bowel. This may trigger periodic aggravation of the disease. In fact, all animal models of colitis described to date require the presence of bacteria in the bowel.[50,51]

Furthermore, our results are relevant for the development of CpG DNA as adjuvants for vaccination in humans. Both endotoxins and CpG DNA are constituents of the complete Freund's adjuvant,[22–24] which is a potent adjuvant in mice but can not be used in humans because of its toxicity. The reason may be the higher LPS sensitivity in humans compared with mice combined with the synergy of CpG DNA and LPS to stimulate toxic inflammatory cytokines. The finding that CpG DNA alone has strong adjuvant activity supports the view that bacterial DNA contributes to the adjuvant activity of the complete Freund's adjuvant.[52] Indeed in mice, CpG ODNs are equally efficient adjuvants compared with the complete Freund's adjuvant to promote specific antibody response.[13] CpG ODN are currently being developed as adjuvants for prophylactic and therapeutic vaccination against infectious disease and tumors in humans.

The presence of CpG motifs contributes to other DNA-based therapeutic strategies. CpG motifs are necessary for effective vaccination with naked DNA.[9] The presence of CpG motifs in antisense ODN may affect their biologic activity.[12] Immune activation by CpG motifs is usually unwanted in gene replacement therapy. However, both viral DNA (adenovirus, retrovirus) and bacteria-derived plasmid DNA used for gene therapy contain different amounts of unmethylated immunostimulatory CpG motifs. In addition, bacteria-derived plasmids usually contain considerable amounts of LPS. The experimental procedure described in this study provides a valuable tool for the quality control of therapeutic DNA preparations with respect to both CpG and LPS.

In conclusion, our study is the first to show the ability of the human immune system to respond to CpG DNA by an activation pattern which is different from LPS. The set of immune responses stimulated by CpG in human cells may be more favorable than the LPS response for therapeutic purposes in terms of lower toxicity and a sustained response. Our results stress the fact that complete elimination of LPS from therapeutic DNA is required in humans, while the presence of LPS might not affect the efficacy of therapeutic DNA in mice because of their lower LPS sensitivity. This is especially important in transferring therapeutic strategies from mouse models to humans.

## Materials and methods

### *Oligodeoxynucleotides and DNA*

Unmodified (phosphodiester) and modified nuclease-resistant (phosphorothioate) ODN were purchased from Operon Technologies (Alameda, CA, USA). The sequences used are: 1760 (20-mer), 5′-ATA ATC GAC GTT CAA GAA AG-3′, completely phosphorothioate-modified to increase nuclease resistance; 1908 (20-mer), 5′-ATA ATA GAG CTT CAA GCA AG-3′, is the corresponding phosphorothioate control non-CpG ODN for 1760; 2059 (24-mer), 5′-TCG TCG TTT TGT CGT TTT GTC GTT-3′, phosphodiester; 2077 (22-mer), 5′-GCT AGC TTT AGA GCT TTA GAG CTT-3′, the corresponding phosphodiester control ODN for 2059. The CpG ODN were used as examples of immune stimulatory sequences. *E. coli* DNA and calf thymus DNA were purchased from Sigma Chemical, St Louis, MO, USA. *E. coli* and calf thymus DNA were made single stranded by boiling for 10 min, followed by cooling on ice for 5 min, before use. DNA samples were diluted in TE-buffer using pyrogen-free reagents.

DNA samples were purified for our initial studies by extraction with phenol-chloroform-isoamyl alcohol (25/24/1) and ethanol precipitation. Where indicated, endotoxin was removed by repeated extraction with Triton x-114 (Sigma Chemical). Before Triton x-114 extraction, 30 μg/ml *E. coli* DNA contained 1.0 EU/ml endotoxin and 30 μg/ml calf thymus DNA 0.14 EU/ml endotoxin (in the LAL assay) (LAL assay; BioWhittaker, Walkersville, MD, USA). After Triton x-114 extraction, endotoxin could no longer be detected in the LAL assay (<0.03 EU/ml for both DNA samples). Purification by Triton x-114 was performed as follows: 25 mg DNA were

diluted in 40 ml pyrogen-free water, 400 µl Triton x-114 was added and the solution mixed at 4°C for 15 min and placed on ice for an additional 20 min. Subsequently, the solution was incubated for 25 min at 55°C (cloudy upper layer and clear lower Triton x-114 layer) and centrifuged for 20 min (600 *g*, 37°C). The upper phase was transfered to a new tube, 400 µl Triton x-114 was added, and previous steps were repeated until the DNA was found to be endotoxin-free by intracellular TNF staining and flow cytometry as described below.

### *Detection of endotoxin*

The activity of LPS is standardized by the FDA using the limulus amebocyte lysate (LAL) assay (EU/ml). The lower detection limit of the LAL assay is 0.1 EU/ml. The measurement of concentrations between 0.1 and 0.01 EU/ml is possible but shows variability. The LPS sample used in our studies (from *Salmonella typhimurium*, Sigma Chemical) had an activity of 4.35 ng/EU in a standardized LAL assay. In our hands the lower detection limit of the LAL assay was 0.03 EU/ml which correlates to 131 pg/ml of our LPS sample. However, 25 pg/ml LPS stimulated considerable amounts of IL-6 in PBMC. Using flow cytometry and intracellular TNF staining (see section on Intracellular staining of TNF and IL-6), the lower detection limit of LPS was 6 pg/ml (0.0014 EU/ml). Where indicated, all ODN and DNA samples have been purified until no endotoxin could be detected by flow cytometry and TNF staining (below 6 pg/ml or 0.0014 EU/ml).

### *Cell preparation and cell culture*

Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers by Ficoll–Paque density gradient centrifugation (Histopaque-1077, Sigma Chemical) as described.[53] Cells were suspended in RPMI 1640 culture medium supplemented with 10% (v/v) heat-inactivated (56°C, 1 h) FCS (HyClone, Logan, UT, USA), 1.5 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco BRL, Grand Island, NY, USA) (complete medium). All compounds were purchased endotoxin-tested. Viability was determined before and after incubation with ODN by trypan blue exclusion (conventional microscopy) or by propidium iodide exclusion (flow cytometric analysis). In all experiments, 96 to 99% of cells were viable.

Freshly prepared PBMC (final concentration $1 \times 10^6$ cells/ml) were cultured in complete medium in the presence of different stimuli in a 5% $CO_2$ humidified incubator at 37°C. Cell cultures were performed in 48-well plates (800 µl) or 96-well plates (200 µl). At the indicated time-points, supernatants for the IL-6 ELISA were removed and kept at –20°C, or cells were harvested for flow cytometry as described below.

### *Surface antigen staining*

All antibodies used were purchased from Pharmingen, San Diego, CA, USA. Surface antigen staining and analysis by flow cytometry was performed as previously described.[54] To identify monocytes in PBMC, a FITC-labeled mouse anti-human mAb against CD14 (M5E2) was used. ICAM-1 expression was examined using a phycoerythrin (PE)-labeled mouse anti-human mAb against ICAM-1 (HA58). FITC-labeled $IgG_1$, κ (MOPC-21) and PE-labeled $IgG_{2b}$, κ (27–35) were used to control for specific staining.

### *Intracellular staining of TNF and IL-6*

For intracellular cytokine staining, PBMC (final concentration $1 \times 10^6$ cells/ml) were incubated in the presence of brefeldin A (final concentration 1 µg/ml, stock solution 1 mg/ml in ethanol, Sigma Chemical) to block protein secretion during the indicated time spans. After incubation, cells were harvested and resuspended in 100 µl of buffer A (Fix and Perm Kit, Caltag Laboratories, Burlingame, CA, USA), incubated at room temperature for 15 min, 2 ml PBS was added and cells were centrifuged (400 *g*, 5 min, 4°C). The supernatant was removed completely and cells were resuspended in 100 µl buffer B (Fix and Perm Kit) and 10 µl of either the PE-labeled mouse anti-human TNF mAb (MAb11) or the PE-labeled rat anti-human IL-6 mAb (MQ2-6A3). After 15-min incubation at room temperature in the dark, 2 ml PBS was added and the cells were centrifuged (400 *g*, 5 min, 4°C). Cells were resuspended in 100 µl ice-cold PBS and 10 µl of a FITC-labeled mouse anti-human CD14 mAb (M5E2) and incubated on ice in the dark for 15 min. Cells were washed with 2 ml ice-cold PBS and examined by flow cytometry.

### *Flow cytometry*

Flow cytometric data of 5000 cells per sample (cells from the morphologic monocyte gate only) were acquired on a FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA). In two and three color flow cytometric analyses, spectral overlap was corrected by appropriate compensation. Fluorescence detector settings were adjusted, so that stained cells were on scale for each parameter. Non-viable cells were excluded from analysis by propidium iodide staining (2 µg/ml). Data were analyzed using the computer program FlowJo (version 2.5.1, Tree Star, Stanford, CA, USA).

### *ELISA for IL-6*

Flat-bottom Immulon-1 plates (Dynatech Laboratories, Chantilly, VA, USA) were coated with 100 µl per well (4 µg/ml) of anti-human IL-6 mAb (6708.111, R&D Systems, Minneapolis, MN, USA) diluted in PBS overnight at room temperature. The plates were washed with 0.05% Tween 20 in PBS, pH 7.4, and blocked by incubating with 300 µl per well of PBS containing 1% BSA, 5% sucrose and 0.05% $NaN_3$ for 1 h at room temperature. After washing, 100 µl of cell culture supernatants or recombinant human IL-6 (PharMingen) appropriately diluted in diluent buffer (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline pH 7.3) were added in duplicates and incubated for 2 h at room temperature. Plates were washed, 100 µl per well of the biotinylated mouse anti-human IL-6 mAb (R&D Systems) were added and plates were incubated for an additional 2 h followed by washes. Horseradish peroxidase-conjugated streptavidin (100 µl per well) (Zymed, San Francisco, CA, USA) at a 1/20 000 dilution in diluent buffer was added and incubated at room temperature for 20 min followed by three washes. The plates were developed with 3,3',5,5'-tetramethyl-benzidine (TMB, Sigma Chemical) (100 µl per well) for 20 min. The reaction was stopped with 0.67 N $H_2SO_4$ and plates were read on a microplate reader (Cambridge Technology, Watertown, MA, USA) set to 450 nm with wavelength

correction to 570 nm. The lower detection limit was 10 pg/ml.

### *Statistical analysis*

Data were expressed as means ± s.e.m. Statistical significance of differences was determined by the unpaired two-tailed Student's *t* test. Differences were considered statistically significant for $P < 0.05$. Statistical analyses were performed by using StatView 4.51 software (Abacus Concepts, Calabasas, CA, USA).

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The Journal of Immunology



# Phosphorothioate Oligodeoxynucleotides Promote the In Vitro Development of Human Allergen-Specific CD4+ T Cells into Th1 Effectors

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# Phosphorothioate Oligodeoxynucleotides Promote the In Vitro Development of Human Allergen-Specific CD4$^+$ T Cells into Th1 Effectors[1]

**Paola Parronchi, Francesca Brugnolo, Francesco Annunziato, Cinzia Manuelli, Salvatore Sampognaro, Carmelo Mavilia, Sergio Romagnani,[2] and Enrico Maggi**

**DNA vaccination is an effective approach in inducing the switch of murine immune responses from a Th2 to a Th1 profile of cytokine production that has been related to the activity of unmethylated CpG motifs present in bacterial, but not mammalian, DNA. We report here that some synthetic phosphorothioate, but not phosphodiester, oligodeoxynucleotides (ODNs) were able to induce B cell proliferation and to shift the in vitro differentiation of *Dermatophagoides pteronyssinus* group 1-specific human CD4$^+$ T cells from atopic donors into Th cell effectors showing a prevalent Th1, instead of Th2, cytokine profile. This latter effect was completely blocked by the neutralization of IL-12 and IFN ($\alpha$ and $\gamma$) in bulk culture, suggesting that the Th1-inducing activity of phosphorothioate ODNs was mediated by their ability to stimulate the production of these cytokines by monocytes, dendritic, and NK cells. Cytosine methylation abolished the Th1-inducing activity of ODNs; however, CpG dinucleotide-containing ODNs exhibited the Th1-shifting effect independently of the presence or the absence of CpG motifs (5′-pur-pur-CpG-pyr-pyr-3′). Moreover, the inversion of CpG to GpC resulted only in a partial reduction of this activity, suggesting that the motif responsible for the Th1-skewing effect in humans is at least partially different from that previously defined in mice. These results support the concept that the injection of allergens mixed to, or conjugated with, appropriate ODNs may provide a novel allergen-specific immunotherapeutic regimen for the treatment of allergic disorders. *The Journal of Immunology*, 1999, 163: 5946–5953.**



Allergic disorders in humans are characterized by a prevalent Th2 effector response to "innocuous" environmental Ags (allergens) (1–3). Allergen-specific Th2 cells produce IL-4 and IL-13, which are responsible for the IgE isotype switching (4–6), IL-10, which, like as IL-4, favors the growth of mast cells (7, 8), and IL-5, which promotes the differentiation, activation, and in situ survival of eosinophils (9, 10). Thus, allergen-specific Th2 responses account for the joint involvement of IgE-producing B cells, mast cells, and eosinophils in the allergic inflammation (reviewed in Ref. 11).

Immunization with Ag-encoding plasmid DNA has generated great interest, especially for its ability to provide a highly favorable microenvironment for the preferential development of Ag-reactive T cells into Th1 effector cells (12). Therefore, DNA vaccination has been attempted, and proved to be an effective approach, in altering the allergen-specific IgE responses in mice (13, 14). However, the effects of DNA allergen gene vaccination in humans are presently unknown, as factors contributing to DNA vaccine immunogenicity have not yet been fully clarified and the possible side effects of this type of vaccination remain, at least partially, to be defined. Recently, several reports have shown that the ability of DNA vaccines to favor the development of Th1 responses in experimental animal models is mainly due to unmethylated "CpG motifs" (5′-pur-pur-CpG-pyr-pyr-3′), which are present in bacterial, but not mammalian, DNA (12, 15). These compounds are indeed able to stimulate APC and NK cells to produce a series of immunomodulatory cytokines, including IL-12, IFN-$\alpha$, IFN-$\gamma$, and IL-18 (16–19), which induce the development of both Th1 cells and Th1-dependent cytotoxic T cell responses (20–23), as well as IL-6 (24), which promotes B cell activation and Ab secretion (25). Accordingly, coadministration with the Ag of oligodeoxynucleotides (ODNs)[3] containing the CpG-motif (CpG-ODNs) prevented airway eosinophilia, Th2 cytokine induction, IgE production, and bronchial hyperreactivity in murine models of asthma (26–29).

In this study, we have tested the activity of a series of synthetic ODNs on both human B cell proliferation and in vitro development of *Dermatophagoides pteronyssinus* group 1 (Der p 1)-specific T cells from atopic Der p-sensitive donors. Phosphorothioate (PS), but not phosphodiester (PE), ODNs induced not only B cell proliferation but also a dose-dependent switch from a prevalent Th2 to a prevalent Th1 cytokine profile in allergen-specific short-term T cell lines, as detected by both the cytofluorometric analysis of intracellular cytokine synthesis at single-cell level and the measurement of IFN-$\gamma$ and IL-4 concentrations released in their supernatants. In agreement with the results reported in experimental animal models (19–20), the Th1-inducing activity of CpG-containing PS-ODNs on allergen-specific human T cells was due to their ability to induce the production of Th1-inducing cytokines, as their effect was completely inhibited by the addition in culture of a mixture of anti-IL-12 and anti-IFNs Abs. Although cytosine methylation abolished the ODN-mediated activity, ODNs lacking

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[3] Abbreviations used in this paper: ODN, oligodeoxynucleotide; PS, phosphorothioate; PE, phosphodiester; Der p 1, *Dermatophagoides pteronyssinus* group 1; MI, mitogenic index.



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Table 1. *Sequence, length, and source of ODNs used in the study*

| Name | Sequence[a] | Length | Source |
|---|---|---|---|
| 3Da | GAG *AAC GCT* CGA CCT TCC AT | 20 mer | |
| DSP30 | TCG TCG CTG TCT CCG CTT CTT CTT GCC | 27 mer | Antisense of HIV-rev |
| DSP30/GC | TGC TGC CTG TCT GCC CTT CTT CTT GCC | 27 mer | |
| DSP17 | GCC GAG CTC CAT CTC GTA CGC | 21 mer | Antisense of HSV-orf |
| 2105 | TTG CTT CCA TCT TCC TCG TC | 20 mer | From Papilloma virus |
| Myco | GTG CTC GAG GAT GCG CTT CGC | 21 mer | From *Mycobacterium tuberculosis* |
| 1326 | *CAG CGC TCC CGT CGC* TCT CTC | 21 mer | From CD30 gene |
| Poly CG | CGC GCG CGC GCG CGC GCG CGC G | 22 mer | |

[a] Italics indicates "CpG motifs" (5'-pur-pur-CpG-pyr-pyr-3'). Underlining indicates CpG dinucleotides.

the entire CpG motif also exerted a maximal effect, whereas the inversion of CpG dinucleotides to CpG resulted only in a partial reduction of the Th1-inducing activity. These findings suggest that, differently from mouse models, CpG motifs and CpG dinucleotides may not be necessarily required to evoke the ODN-mediated effects in humans.

## Materials and Methods

### *Subjects*

Blood samples used in this study were obtained from 10 informed adult atopic Der p-sensitive volunteers and four healthy volunteers in accordance with the ethical standards of the responsible regional committee on human experimentation.

### *Reagents*

The medium used throughout was RPMI 1640 (Seromed, Berlin, Germany) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, $2 \times 10^{-5}$ M 2-ME (all from Life Technologies, Grand Island, NY), 100 $\mu$g/ml kanamycin, and 10 $\mu$g/ml gentamicin (Sigma, St. Louis, MO) (complete medium). Der p 1 allergen was kindly provided by Lofarma SpA (Milan, Italy). PMA, ionomycin, brefeldin A, and saponin were all purchased from Sigma. IL-2 was a kind gift from Eurocetus (Milan, Italy), and IL-12 was a kind gift from G. Trinchieri (Wistar Institute, Philadelphia, PA). Purified and fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16, anti-CD20, and anti-CD56 mAbs were purchased from Becton Dickinson (San Jose, CA). Phycoerythrin-conjugated anti-IL-4 (3010.211, IgG1) and FITC-conjugated anti-IFN-$\gamma$ (25723.11, IgG2b) were purchased from Becton Dickinson (San Diego, CA). Purified and fluorochrome-conjugated isotype control mAbs were purchased from Southern Biotechnology Associates (Birmingham, AL).

### *Synthetic ODNs*

The eight ODNs used in this study, listed in Table I, were obtained from Genset (Paris, France). They were all HPLC-purified and free for endotoxin contamination. PE- and PS-ODNs, as well as PS-ODNs in which all cytosines were methylated (PS/met-ODNs), with identical base sequence, were used. The sequences 3Da, DSP30, DSP17, and 2105 have been already described and used by others (25, 30). The sequences Myco, 1326, and poly CG were assessed for the first time in this study. The sequence PS-DSP30, in which all the CpG dinucleotides were inverted in GpC (PS-DSP30/GC), was also used. CpG motifs present in the different ODNs are indicated in italic, whereas CpG dinucleotides are underlined.

### *B cell proliferation*

PBMC from healthy volunteers were isolated by Ficoll-Hypaque gradient centrifugation and depleted of T cells, NK cells, and macrophages by treatment with anti-CD3, anti-CD16, and anti-CD14 mAbs ($2\ \mu g/10^7$ cells) followed by the addition of microbead-conjugated goat anti-mouse IgG (Miltenyi Biotec, Bisley, Germany). Cells were then separated on magnetic cell separation system columns (Miltenyi Biotec) following manifacturer's instructions. Highly purified B cell populations (containing <1% non-B cells) were seeded at the concentration of $1 \times 10^6$/ml in triplicate in U-bottomed well plates (Nunc, Nunclon, Denmark) in complete medium and 10% heat-inactivated FCS (HyClone, Logan, UT) in a 0.2-ml volume for 48 h in the absence or presence of different concentrations of ODNs (10, 5, and 0.5 $\mu$g/ml). After 16 h pulsing with 0.5 $\mu$Ci [$^3$H]TdR per well (Amersham, Little Chalfont, U.K.), cultures were harvested and radionuclide uptake measured by scintillation counting.

### *Production of proinflammatory cytokines by non-T cells*

PBMC from healthy volunteers were isolated by Ficoll-Hypaque and then enriched for non-T cells by a rosetting technique using neuroaminidase-treated SRBC. Non-T cells ($1 \times 10^6$) were then seeded in 48 flat-bottom well plates (Costar, Corning, NY) in 1 ml complete medium plus 10% heat-inactivated FCS in the absence or presence of PS- or PE-DSP30 (10 $\mu$g/ml). After 72 h incubation, supernatants were collected, centrifuged, and stored at −20°C. The amounts of IL-12, IFN-$\alpha$, IL-6, and IL-1 receptor antagonist were evaluated by specific commercial ELISAs (Endogen, Woburn, MA and R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

### *Generation of short-term Der p 1-specific T cell lines*

Der p 1-specific $CD4^+$ T cell lines were generated as previously described (31). Briefly, PBMC were obtained from 10 atopic Der p-sensitive donors by centrifugation on Ficoll-Hypaque gradient and stimulated with Der p 1 (10 $\mu$g/ml) for 6 days in complete RPMI 1640, containing 5% heat-inactivated autologous serum, in the absence or presence of different concentrations of PS- or PE-, or PS/met-ODNs, or rIL-12 (100 U/ml). On day 6, activated T cells were expanded for subsequent 8 days by the addition of IL-2 (20 U/ml). In the inhibition experiments, neutralizing anti-IL-12 mAb alone (R&D Systems) or a mixture of anti-IL-12, anti-IFN-$\alpha$ (Biosource, Camarillo, CA), and anti-IFN-$\gamma$ (R&D Systems) mAbs were added at the beginning of the culture, at a concentration of 10 $\mu$g/ml. In parallel cultures, control isotype mAbs were used (Southern Biotechnology Associates).

The specificity of short-term T cell lines was assessed as already described (31). Briefly, $5 \times 10^4$ T cell blasts were incubated in the presence of $5 \times 10^4$ autologous irradiated PBMC, as APC, and allergen (Der p 1, 10 $\mu$g/ml) for 48 h in a 0.2-ml volume in duplicate. After a 16-h pulse with 0.5 $\mu$Ci [$^3$H]TdR (Amersham), cultures were harvested and radionuclide uptake was measured by scintillation counting. T cell lines were considered as specific when mitogenic index (MI) was ≥5.

### *Intracytofluorometric analysis of cytokine production*

Intracytofluorometric analysis of IFN-$\gamma$ and IL-4 synthesis at the single-cell level was performed as described (31, 32). Briefly, $1 \times 10^6$ T cell blasts were stimulated with PMA (10 ng/ml) plus ionomycin (1 $\mu$M) for 4 h, the last two of which was in the presence of brefeldin A (5 $\mu$g/ml). After incubation, cells were washed twice with PBS, pH 7.2, fixed 15 min with formaldehyde (2% in PBS, pH 7.2), washed twice with 0.5% BSA in PBS, pH 7.2, permeabilized with PBS, pH 7.2, containing 0.5% BSA and 0.5% saponin, and then incubated with the specific mAbs. Cells were analyzed on a FACScalibur cytofluorometer using the CellQuest software (Becton Dickinson). The area of positivity was determined using an isotype-matched mAb. In all cytofluorometric analyses, a total of $10^5$ events, gated as $CD3^+CD4^+$ or $CD3^-CD16^+$ cells, for each sample, were acquired.

### *Cytofluorometric analysis of cell-surface Ags*

To evaluate the percentages of NK cells in Der p 1-specific T cell lines obtained in the absence or presence of ODNs, $2 \times 10^5$ cells were collected at the time of the cytokine assessment and stained with anti-CD3, anti-CD16, anti-CD20, anti-CD14, and anti-CD56 fluorochrome-conjugated mAbs (Becton Dickinson) or with appropriate isotype control mAbs. After

Table II. *PS-, but neither PE- nor PS/met-, ODNs stimulate human B cell proliferation*[a]

| Reagent Added in Bulk Culture (10 μg/ml) | B Cell Proliferation (MI)[b] | | | | | |
|---|---|---|---|---|---|---|
| | Expt. 1 | | | Expt. 2 | | |
| | PE | PS | PS/met | PE | PS | PS/met |
| DSP30 | 1.1 | 10.0 | 2.1 | 0.9 | 27.0 | 4.0 |
| DSP17 | 1.3 | 13.0 | 2.0 | 0.7 | 21.0 | 3.5 |
| 3Da | 0.9 | 6.5 | 1.8 | 1.1 | 27.8 | 4.2 |
| 2105 | 1.0 | 6.0 | 3.0 | 1.1 | 17.0 | 5.0 |
| Myco | 0.8 | 10.4 | 2.3 | 1.3 | 25.0 | 7.5 |
| 1326 | 1.0 | 7.0 | 1.4 | 0.9 | 22.5 | 4.0 |
| Poly CG | 1.1 | 1.2 | 1.0 | 0.7 | 1.1 | 0.9 |

[a] Highly purified B cells from two healthy volunteers were stimulated for 2 days in the presence or absence of PE-, PS-, or PS/met-ODNs (10 μg/ml). After 16 h pulsing with [$^3$H]TdR, cultures were harvested and radionuclide uptake measured by scintillation counting, as described in *Materials and Methods*.

[b] MI was calculated as the ratio between the mean values of cpm obtained in ODN-stimulated cultures and cpm obtained in the presence of medium alone.

two washings, cells were resuspended and analyzed by flow cytometry with the use of the FACScan system (Becton Dickinson). A total of $10^4$ cells for each sample were acquired.

### *Measurement of IL-4 and IFN-γ in the supernatants of Der p 1-specific T cell lines*

The ability of Der p 1-specific T cell lines to produce cytokines was evaluated following stimulation of $10^6$/ml viable T cell blasts with $10^6$/ml autologous irradiated PBMC, as APC, and Der p 1 10 μg/ml in a 1-ml volume for 72 h. IL-4 (PharMingen, San Diego, CA) and IFN-γ (Endogen) concentrations were measured into cell-free supernatants by homemade ELISAs using commercial pairs of mAbs, as previously described (31).

### *Statistical analysis*

Statistical analysis of the results was performed by Student's *t* test.

## Results

### *ODNs induce human B cell proliferation*

In a first series of experiments, all ODNs listed in Table I were assessed for their ability to induce the proliferation of human B cells. To this end, purified peripheral blood B cells derived from two healthy donors were incubated for 3 days with different concentrations of PS-ODNs, the corresponding PE- or PS/met-ODNs, and [$^3$H]thymidine incorporation was assessed. As shown in Table II, all PS-ODNs (3Da, DSP30, DSP17, and 2105), already reported to act as powerful activators for murine (3Da) (25) or human (DSP30, DSP17, 2105) (30) B cells, as well as two PS-ODNs not previously assessed (Myco and 1326), induced remarkable B cell proliferation, whereas both the corresponding PE- and PS/met-ODNs did not. Table II also shows that poly CG was ineffective in inducing human B cell proliferation.

### *ODNs favor the in vitro development of allergen-specific T cells into Th1-like effectors*

To investigate the effects of ODNs on the in vitro development of allergen-specific human T cells, two of the above mentioned PS-ODNs (PS-DSP30 and PS-DSP17) were initially used. To this end, Der p 1-specific short-term T cell lines were generated from PBMC of two atopic Der p-sensitive donors in the absence or presence of three different concentrations (0.5, 5, and 10 μg/ml) of PS-DSP30 and PS-DSP17 and assessed by flow cytometry for intracellular synthesis of IFN-γ and IL-4 following polyclonal stimulation with PMA and ionomycin. As positive control, the effects exerted on parallel cultures by IL-12 (a powerful Th1 inducer) (33), were evaluated. As shown in Table III, there was a dose-dependent increase in the proportion of cells expressing IFN-γ and a reduction in the proportion of cells expressing IL-4 in cultures conditioned with either PS-DSP30 or PS-DSP17 in comparison with unconditioned cultures. The shift to the Th1 cytokine profile observed in PS-ODN-conditioned cultures was similar to that observed in IL-12-conditioned cultures. Similar results were obtained by establishing Der p 1-specific T cell lines from PBMC of the same donors in the absence or presence of other PS-ODNs, such as 3Da, 2105, Myco, and 1326, all used at the concentration of 10 μg/ml (Table III).

In subsequent experiments, the activity of PS-DSP30, used at a fixed concentration (10 μg/ml), was assessed on Der p 1-specific T cells from a higher number of atopic donors (eight subjects total). As additional controls, parallel Der p 1-stimulated cultures were also established in the presence of PE-DSP30 (10 μg/ml) or

Table III. *Effects of PS-ODNs on the cytokine profile of human Der p 1-specific short-term T cell lines*[a]

| Reagents Added in Bulk Culture | Concentration (μg/ml) | % of Cells Expressing the Indicated Cytokine | | | | | |
|---|---|---|---|---|---|---|---|
| | | Expt. 1 | | | Expt. 2 | | |
| | | IL-4$^+$ | IFN-γ$^+$ | IL-4$^+$ IFN-γ$^+$ | IL-4$^+$ | IFN-γ$^+$ | IL-4$^+$ IFN-γ$^+$ |
| Medium | | 34.2 | 12.6 | 10.1 | 20.9 | 21.4 | 15.4 |
| rIL-12 | | 6.1 | 35.7 | 19.1 | 6.7 | 34.1 | 16.1 |
| PS-DSP30 | 0.5 | 34.3 | 13.7 | 9.4 | 24.5 | 22.0 | 25.7 |
| | 5.0 | 14.9 | 29.7 | 17.9 | 6.3 | 31.5 | 10.2 |
| | 10.0 | 18.5 | 24.6 | 23.9 | 5.9 | 34.6 | 8.2 |
| PS-DSP17 | 0.5 | 32.2 | 16.7 | 11.9 | 17.9 | 27.4 | 14.4 |
| | 5.0 | 18.5 | 20.8 | 12.1 | 5.0 | 30.5 | 4.4 |
| | 10.0 | 21.5 | 19.9 | 15.6 | 7.0 | 30.9 | 5.5 |
| PS-3Da | 10.0 | 16.3 | 27.1 | 18.3 | 6.5 | 30.8 | 8.2 |
| PS-2105 | 10.0 | 22.2 | 24.4 | 16.5 | 6.4 | 33.2 | 5.4 |
| PS-Myco | 10.0 | 21.5 | 19.8 | 16.0 | 5.4 | 35.8 | 6.6 |
| PS-1326 | 10.0 | 18.0 | 25.0 | 22.0 | 8.1 | 38.7 | 17.8 |

[a] PBMC from two atopic Der p-sensitive donors were stimulated for 6 days with Der p 1 (10 μg/ml) in the presence of medium alone, IL-12 (100 U/ml), or different concentrations of PS-ODNs, as indicated. T cell blasts were then expanded for subsequent 8 days with IL-2 and then assessed for their IL-4 and IFN-γ intracellular content by flow cytometry, following stimulation with PMA and ionomycin for 4 h, the last two being in the presence of brefeldin A, as described in *Materials and Methods*.

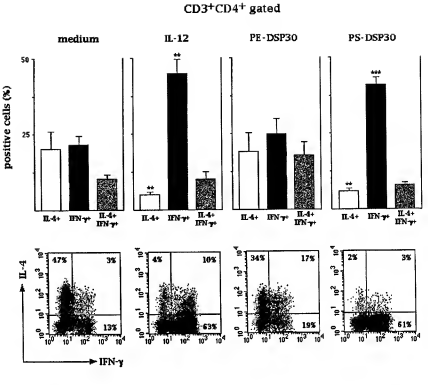


**FIGURE 1.** Effect of PS-ODN on intracellular IL-4 and IFN-γ expression by Der p 1-specific T cells. Der p 1-specific short-term T cell lines were generated from PBMC of eight atopic Der p-sensitive donors in the absence or presence of IL-12 (100 U/ml), PE-DSP30, or PS-DSP30 (10 μg/ml). After 14 days, T cell blasts were stimulated with PMA plus ionomycin, as described in *Materials and Methods*, and intracellular cytokine synthesis was evaluated in $CD3^+$ $CD4^+$ T cells by cytofluorometric analysis. In the *upper panel*, columns represent the mean values (±SE) of cells expressing IL-4 alone (□), IFN-γ alone (■), or both IL-4 and IFN-γ (▨) (**, $p < 0.005$; ***, $p < 0.0001$). In the *lower panel*, the cytofluorometric patterns of $CD3^+$ $CD4^+$ Der p 1-specific T cell blasts from one representative donor are shown.

IL-12 (100 U/ml). The results of these experiments are summarized in Fig. 1 (*upper panel*). A significant increase in the proportion of IFN-γ-producing T cells and a significant decrease in the proportion of IL-4-producing T cells were observed when cultures were conditioned with PS-DSP30 ($p < 0.0001$ and $<0.005$, respectively) or IL-12 ($p < 0.005$) in comparison with either unconditioned cultures or cultures conditioned with PE-DSP30. By contrast, the proportion of cells able to produce both IFN-γ and IL-4 was not significantly different in all types of cultures. The cytofluorometric profile observed in one representative experiment is shown in Fig. 1 (*lower panel*).

To provide undoubtable evidence that the Th1-shifting effect of ODNs was indeed exerted on allergen-specific T cells, Der p 1-specific short-term T cell lines, generated in the presence of medium alone, PS-DSP30, PE-DSP30, or IL-12, were stimulated for 72 h with Der p 1 and APC under MHC-restricted conditions. IL-4 and IFN-γ concentrations were then measured by appropriate ELISAs into cell-free supernatants. Even under conditions of specific Ag-stimulation, a lower IL-4 production in PS-DSP30-conditioned than unconditioned, or PE-DSP30-conditioned, T cell cultures was observed ($p < 0.05$). Moreover, the addition to the cultures of PS-DSP30 resulted in a highly significant increase in the IFN-γ production by Der p 1-stimulated T cells in comparison with unconditioned cultures, or cultures conditioned with PE-DSP30 ($p = 0.007$) (Fig. 2).

### *The Th1-inducing effect of ODNs is due to their ability to stimulate the production of IFNs and IL-12 by cells of the innate immunity*

The possibility that the Th1 shift in the development of Der p 1-specific T cells induced by PS-ODNs was due to their ability to promote the production of Th1-inducing cytokines was then investigated. To this end, in a first series of experiments, T cell-depleted PBMC suspensions from two healthy subjects were incubated for 3 days with medium alone, PS-DSP30, or PE-DSP30 (10 μg/ml), and concentrations of IFN-α, IL-12, IL-6, and IL-1

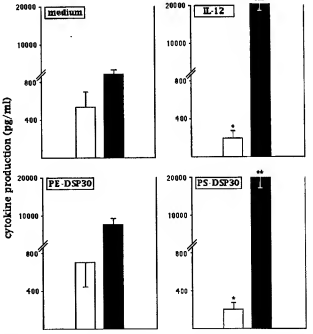


**FIGURE 2.** Effect of PS-ODN on Der p 1-stimulated production of IL-4 and IFN-γ by Der p 1-specific T cells. Der p 1-specific short-term T cell lines were generated from PBMC of eight atopic Der p-sensitive donors in the absence or presence of IL-12 (100 U/ml), PE-DSP30, or PS-DSP30 (10 μg/ml). After 14 days, $10^6$ T cell blasts were stimulated for 72 h with Der p 1 (10 μg/ml) in the presence of $10^6$ irradiated autologous PBMC, as APCs. The content of IL-4 (□) and IFN-γ (■) (±SE) was measured into cell-free supernatants by specific ELISAs, as described in *Materials and Methods*. (*, $p < 0.05$; **, $p < 0.007$; ***, $p < 0.005$)

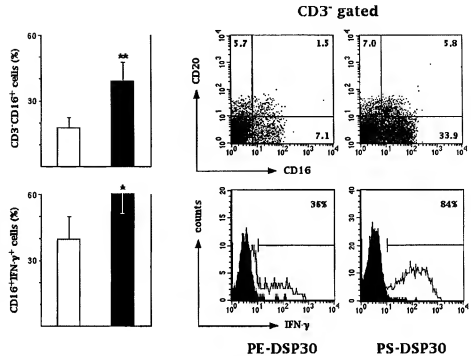


**FIGURE 3.** PS-ODNs expand higher proportions of $CD3^-CD16^+$ IFN-γ-expressing cells in Der p 1-stimulated cultures. Der p 1-specific short-term T cell lines were generated from eight atopic Der p-sensitive donors and, at the time of cytokine assessement, cells were stained with anti-CD3, anti-CD16, and anti-CD20 mAbs. Mean percentages (±SE) of $CD3^-CD16^+$ cells present in PE-DSP30-conditioned (□) or PS-DSP30-conditioned cultures (■) are reported in the *left* of the *upper panel*, whereas the cytofluorometric analysis from a representative experiment is shown on the *right*. Mean percentages (±SE) of $CD3^-CD16^+$ cells expressing IFN-γ after stimulation with PMA plus ionomycin in cultures derived from the same atopic donors and conditioned with PE-DSP30 (□) or PS-DSP30 (■) are reported in the *left* of the *lower panel*, whereas hystograms from a representative experiment are shown on the *right*. (*, $p = 0.008$; **, $p < 0.005$)

receptor antagonist released in their supernatants were measured. Only non-T cells incubated in the presence of PS-DSP30 showed a detectable increase in the production of these cytokines (data not shown). Subsequently, to determine whether an expansion of non-T cells could contribute to the Th1-shift observed in PS-ODN-modulated cultures, Der p 1-specific T cell lines generated in the presence of either PS- or PE-DSP30 were assessed for both the proportions and cytokine profile of non-T cells. As shown in Fig. 3 (*upper panel*), a significantly higher proportion of $CD3^-$ $CD16^+$ cells (NK cells) was observed in cultures generated in the presence of PS-DSP30 in comparison with those generated in the presence of PE-DSP30 ($p < 0.005$), whereas the proportions of $CD3^-$ $CD20^+$ cells (B cells) into the same cultures were comparable and low, probably due to the poor viability of B lymphocytes after 14 days of culture. $CD3^-CD16^+$ cells also showed CD56 expression, which confirms their belonging to the NK cell population (data not shown). More importantly, PS-DSP30-conditioned cultures showed significantly higher proportions of $CD3^-CD16^+$ cells able to synthesize intracellular IFN-γ in response to the stimulation with PMA plus ionomycin than PE-DSP30-conditioned cultures (Fig. 3, *lower panel*). No significant difference in the proliferation of PS-DSP30-conditioned cultures in response to Der p 1 plus autologous irradiated PBMC (Ag-specific proliferation) was observed, despite the increased numbers of NK cells (data not shown).

To provide further evidence that the Th1-shifting activity of PS-ODNs was mediated by their ability to induce the production of IL-12 and IFN (α and γ) by cells of the innate immunity (monocytes, dendritic cells, and NK cells), short-term T cell lines were generated from two atopic Der p-sensitive donors by stimulating their PBMC with Der p 1 and PS-DSP30 in the presence of anti-IL-12 neutralizing mAb alone, a mixture of anti-IL-12 and anti-IFN (α and γ) neutralizing mAbs, or isotype-matched control mAbs, respectively. The cytokine profile of Der p 1-specific T cell lines derived under these different experimental conditions was then compared by analyzing the intracellular IL-4 and IFN-γ synthesis at the single-cell level following polyclonal stimulation with PMA and ionomycin. As shown in Fig. 4, the Th1-shifting effect of PS-DSP30 was partially inhibited by the addition of anti-IL-12 mAb, but completely blocked by the mixture of anti-IL-12 and anti-IFNs mAbs. Similar results were obtained when cytokine concentrations were measured in culture supernatants following stimulation with Der p 1 under MHC-restricted conditions (data not shown).

### *The presence of CpG is not necessarily required for the Th1-inducing effect of ODNs*

The data reported above (Tables II and III) demonstrate that not only CpG motif-containing (3Da and 1326), but also CpG motif-lacking (DSP30, DSP17, 2105, and Myco) ODNs were able to induce both the proliferation of human B cells and the Th1-shift of allergen-specific T cells. This suggests that the presence of particular bases flanking CpG dinucleotides may not be necessarily required to promote these effects in humans. Therefore, to better clarify the nature of the molecular structures responsible for the activity of ODNs, the effect of cytosine methylation on the Th1-shifting activity of PS-DSP30 and PS-DSP17 was first assessed. In agreement with the results already reported in experimental animal models (25, 34), methylation completely abolished not only the activity of PS-ODNs on human B cell proliferation (already shown in Table II), but also their Th1-inducing ability (Table IV). In contrast, a PS-repetitive sequence of CpG, $(CpG)_{11}$, such as poly CG, did not affect neither human B cell proliferation (Table II) nor the differentiation of Der p 1-specific T cells (data not shown). Finally, the actual role of CpG dinucleotides as responsible for the effects of PS-ODNs was further investigated, comparing the ability of PS-DSP30 and the same ODN in which CpG dinucleotides were inverted to GpC (PS-DSP30/GC), to induce B cell proliferation and to shift the differentiation of Der p 1-specific T cells toward the Th1 profile. As shown in Fig. 5, the modified ODN retained a remarkable, even if lower, activity on both B cell proliferation (*upper panel*) and induction of Th1 shift in Der p 1-specific T cell lines (*lower panel*).

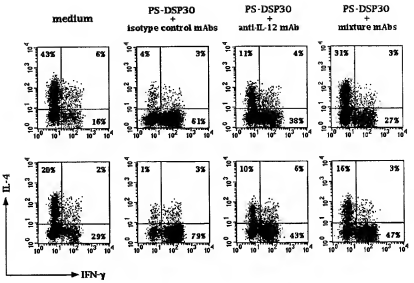


FIGURE 4. Blocking of the Th1-inducing effect of PS-ODN by neutralization of IL-12, IFN-γ, and IFN-α activity. Der p 1-specific short-term T cell lines were generated from two atopic Der p-sensitive donors and conditioned with PS-DSP30 in the presence of neutralizing anti-IL-12 mAb, a mixture of anti-IL-12, anti-IFN-γ, and anti-IFN-α mAbs or isotype control mAbs (10 μg/ml), respectively. After 14 days, T cell blasts were stimulated with PMA plus ionomycin, and intracellular IL-4 and IFN-γ synthesis was evaluated by cytofluorometric analysis on $CD3^+CD4^+$ T cell blasts, as described in *Materials and Methods*.

## Discussion

The immunomodulatory activity of bacterial DNA, originally recognized by examining the effect of CFA, has been extensively investigated in the mouse (12). This activity on the immune system has been ascribed to unmethylated CpG dinucleotides, which are present at a high degree of frequency in bacterial, but not mammalian, DNA (12). More recently, synthetic ODNs containing unmethylated CpG dinucleotides flanked by particular bases (CpG motifs) have been shown to be as powerful as bacterial DNA in triggering the proliferation of B cells and polyclonal Ig secretion in a T cell- and Ag-independent fashion (21, 25), the activation of NK cells (20), and the production of several proinflammatory cytokines (16, 19, 24). In addition, synthetic ODNs have been found to be able to bias the specific immune response to a Th1-dominated cytokine pattern, both in vitro, inasmuch as they are able to stimulate the production of Th1-inducing cytokines, such as IL-12, IFNs, and IL-18 (22, 34), and in vivo, because they have been successfully employed in animal models of Th2-dominated diseases, such as leishmaniasis (23, 35) and asthma (26–29). Thus, these compounds might be used as effective and safe immunomodulators even in established Ag-specific responses.

The results of our study first confirm those previously reported in humans, showing that certain synthetic ODNs were able to induce strong proliferative response by purified peripheral blood B cells. Such an immunostimulatory activity was usually associated with nuclease-resistant PS, but not PE, compounds and was abolished by cytosine methylation (30). More importantly, the results of our study provide the first evidence that the same synthetic ODNs active on B cells are able, at least in vitro, to shift the differentiation of allergen-specific human $CD4^+$ T cells of atopic donors from a prevalent Th0/Th2-like to a prevalent Th1-like profile of cytokine production, whereas the corresponding PE-ODNs are not. The lack of effects of diester ODNs could be due to the shorter half-life in culture (4–6 h) of these compounds because of the rapid degradation by cell nucleases (30). Moreover, the interpretation of the absence of any mitogenic effects on B cells by PE-ODNs could be complicated by the fact that thymidine released by degraded ODNs may compete with [$^3$H]thymidine used in standard proliferation assays. Nevertheless, 20-fold higher concentrations of PE-ODNs (as high as 200 μg/ml) were completely inactive on both B cell proliferation and T cell differentiation. In addition, even when a different enzymatic cell proliferation assay was used, no mitogenic effect on B cells by diester compounds could be observed (data not shown).

The Th1-inducing effect of ODNs appeared to be related to their ability to induce the production of IL-12 and IFN-α by monocytes and/or dendritic cells and possibly the production of IFN-γ by NK cells. First, PS-ODNs, but not the corresponding PE-compounds,

Table IV. *PS-ODNs with methylated cytosine (PS/met ODNs) are ineffective in inducing the Th1 shifting of allergen-specific T cells*[a]

| Reagents Added in Bulk Culture | Preparation | % of Cells Expressing the Indicated Cytokine | | | | | |
|---|---|---|---|---|---|---|---|
| | | Expt. 1 | | | Expt. 2 | | |
| | | $IL\text{-}4^+$ | $IFN\text{-}\gamma^+$ | $IL\text{-}4^+$ $IFN\text{-}\gamma^+$ | $IL\text{-}4^+$ | $IFN\text{-}\gamma^+$ | $IL\text{-}4^+$ $IFN\text{-}\gamma^+$ |
| Medium | | 34.2 | 12.6 | 10.1 | 14.6 | 20.1 | 4.7 |
| IL-12 | | 6.1 | 35.7 | 19.1 | 4.4 | 43.9 | 7.1 |
| DSP30 | PS | 18.5 | 24.6 | 23.9 | 4.7 | 38.8 | 17.8 |
| | PS/met | 31.8 | 20.0 | 13.3 | 8.4 | 18.9 | 1.1 |
| DSP17 | PS | 21.5 | 19.9 | 15.6 | 5.4 | 35.9 | 6.6 |
| | PS/met | 35.5 | 22.2 | 19.8 | 8.1 | 27.5 | 3.1 |

[a] PBMC from two atopic Der p-sensitive donors were stimulated for 6 days with Der p 1 (10 μg/ml) in the presence of medium alone, IL-12 (100 U/ml), or PS- or PS/met ODNs (10 μg/ml). T cell blasts were then expanded for additional 8 days with IL-2 and then assessed for their IL-4 and IFN-γ intracellular content by flow cytometry, following stimulation with PMA and ionomycin for 4 h, the last two of which in the presence of brefeldin A, as described in *Materials and Methods*.

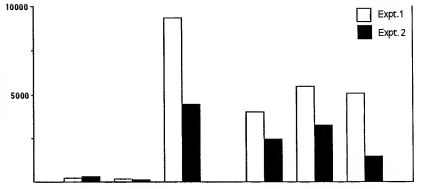


**FIGURE 5.** Effect of CpG inversion to GpC on the ability of PS-DSP30 to stimulate B cell proliferation and to shift the cytokine profile of Der p 1-specific T cells. Highly purified human B cells were obtained from two healthy donors and stimulated in the absence (medium) or presence of different concentrations (10, 5, and 0.5 μg/ml) of PS-DSP30 in which CpG dinucleotides were inverted to GpC (PS-DSP30/GC), as described in *Materials and Methods*. PE- and PS-DSP30 (10 μg/ml) were used in the same experiments as negative or positive controls, respectively (*upper panel*). Der p 1-specific short-term T cell lines were derived from two atopic Der p-sensitive donors in the presence of medium alone, IL-12 (100 U/ml), PE-DSP30, PS-DSP30, or PS-DSP30/GC (10 μg/ml), as described in *Materials and Methods*. After 14 days, T cell blasts were stimulated with PMA plus ionomycin, and intracellular cytokine synthesis was evaluated by flow cytometry (*lower panel*).

| Reagent added in bulk culture | % of $CD3^+CD4^+$ cells expressing the indicated cytokines | | | | | |
|---|---|---|---|---|---|---|
| | Expt.1 | | | Expt.2 | | |
| | IL-4+ | IFN-γ+ | IL-4+ IFN-γ+ | IL-4+ | IFN-γ+ | IL-4+ IFN-γ+ |
| Medium | 37.5 | 14.2 | 15.6 | 37.7 | 3.1 | 7.4 |
| rIL-12 | 6.2 | 49.0 | 11.2 | 17.9 | 28.7 | 53.8 |
| PE-DSP30 | 35.5 | 20.5 | 18.6 | 36.0 | 5.4 | 19.8 |
| PS-DSP30 | 6.3 | 49.5 | 5.6 | 26.7 | 17.9 | 25.2 |
| PS-DSP30/GC | 20.7 | 29.8 | 14.4 | 25.0 | 18.2 | 19.2 |

induced the activation of monocytes and/or dendritic cells, as indicated by the production of small, but detectable over the background, concentrations of IL-12 and IFN-α, by human non-T cells. Second, at the time of cytokine assessment, a significant expansion of $CD3^-CD16^+CD56^+$, IFN-γ-producing cells (NK cells) was observed in PS-ODN-conditioned in comparison with PE-ODN-conditioned or unconditioned cultures. Finally, and more importantly, the Th1-shifting effect of ODNs was partially inhibited by the addition in bulk culture of anti-IL-12 mAb and completely blocked by a mixture of anti-IL-12, anti-IFN-α, and anti-IFN-γ mAbs. These findings indicate that the ability of PS-ODNs to induce the Th1-shift in allergen-specific T cells mainly resides in the stimulation of IL-12 and IFN-α production by monocytes and/or dendritic cells, as well as of IFN-γ production by NK cells. This is consistent with our previous observations showing that cytokines produced by cells involved in the natural immune response against intracellular bacteria and some viruses (IFN-γ, IFN-α, IL-12) may indeed favor the development of the subsequent response of human T cells toward the Th1 effector profile (33, 36, 37). In another study, we showed that the Th1-inducing activity of synthetic dsRNA (poly:I-poly:C) was mediated by its ability to induce the production of both IL-12 and IFN-α by non-T cells (38). IFN-α is indeed critical in the development of human Th1 cells (36, 39) by up-regulating the IL-12 receptor $\beta_2$ chain (40). These data are also in agreement with the results obtained in several animal models showing that CpG-containing ODNs are able to directly stimulate the production of IL-12, IFN-α, IL-18, and IFN-γ by dendritic cells, macrophages, and/or NK cells, which then act as Th1-inducing cytokines (16–19). In contrast, differently from murine models (23), in preliminary experiments no direct effect of PS-ODNs on human T cells could be observed (data not sbown).

The characterization of sequences responsible for the effects on cells of both innate and adaptive immune response in humans is less clear. A large body of evidence, obtained from animals models, indicates that DNA motifs consisting of an unmethylated CpG dinucleotide flanked by two 5′ purines (optimally a GpA) and two 3′ pyrimidines (optimally a TpC or TpT) (CpG motifs) play a fundamental role in conferring the immunostimulatory ability. Indeed, CpG motif-lacking, GpC-inverted, or cytosine-methylated ODNs were ineffective (15, 25). In agreement with the results reported in mice, our findings demonstrate that the Th1-shifting effect of ODNs requires phosphorothioation and is abolished by cytosine methylation. However, differently from the results obtained in the murine experimental models, several sequences were able to stimulate B cell proliferation and to modulate the functional profile of allergen-specific T cells. We found indeed that all the PS-non-repetitive sequences were effective, irrespective of the presence (3Da and 1326) or absence (DSP30, DSP17, 2105, and Myco) of the CpG-motifs previously defined in mice. Actually, a CpG motif-lacking ODN, such as DSP30, exerted the maximal activity. Moreover, CpG dinucleotides per se did not appear to be sufficient, because a PS-repetitive sequence of CpG (poly CG), of similar length to the other ODNs, was completely ineffective. Of note, this type of repeated CG sequence has recently been reported to constitute an inhibitory motif (41). Finally, the PS-DSP30 ODN, in which CpG dinucleotides were inverted to GpC (PS-DSP30/GC), retained its immunomodulatory activity, even if to a lower extent. This suggests that CpG dinucleotides may be important to obtain the maximal effect, but they are not necessarily required to induce an immunomodulatory activity in human T cells.

These findings are consistent with the results reported by Liang et al. (30) with regard to the ability of a variety of ODNs to stimulate the proliferation and differentiation of human B cells. These authors clearly sbowed that, although CpG-containing ODNs are the best stimulators of the B cell response, both CpG motif- and CpG dinucleotide-lacking ODNs possess the ability to induce human B cell activation. Thus, it can be concluded that the immunostimulatory sequences active on murine or human lymphocytes may be, at least partially, different. At present, the nature of the immunostimulatory sequences active in humans remains unclear.

Certainly, nonrepetitive sequences (only one or two bases) are necessary, together with a sufficient length. However, in the absence of a well-recognized sequence, such as the CpG motif in mice, additional studies are required to disclose the 'motif' responsible for the immunomodulatory activity on human B and T cells. Despite this still unsolved problem, the results of the present study provide additional support to the possibility, emerged from the murine models of asthma (13, 27–29), that allergen gene DNA vaccination or injection of allergen mixed to, or modified by the conjugation with, appropriate ODNs may provide a new immunotherapeutic strategy for the treatment of human allergic disorders.

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OLIGONUCLEOTIDES 13:491–499 (2003)


# P-Chirality-Dependent Immune Activation by Phosphorothioate CpG Oligodeoxynucleotides

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## ABSTRACT

**Many of the biologic activities of phosphorothioate oligodeoxynucleotides (PS-oligos) are affected by the sense of chirality of the phosphorus atoms of the internucleotide linkages. Some of the activities are increased by the Rp stereoisomer, and others are increased by the Sp stereoisomer. In previous studies, we showed that PS-oligos containing unmethylated CpG dinucleotides in particular sequence contexts can stimulate B cells and other immune cells. These CpG PS-oligos trigger mitogen-activated protein kinase (MAPK) signaling pathways, causing the induction of B cell proliferation and cytokine and immunoglobulin secretion. We investigated whether the immune stimulation by CpG PS-oligos depends on the sense of their P-chirality. CpG PS-oligos synthesized with internucleotide phosphorothioates of Rp configuration at P-atom showed much stronger MAPK activation and induction of IκB degradation after 40 minutes of stimulation compared with PS-oligos synthesized with Sp linkages. In order to determine if the enhanced stimulatory effects of the Rp stereoisomer may result from differential cellular uptake, we examined the rates at which fluorescently labeled Rp or Sp CpG PS-oligos were taken up by B cells, but these were found to be identical to each other and to stereorandom PS-oligos. The stronger stimulatory effect of the R stereoisomer did not last for 48 hours, and $^{3}$H-thymidine incorporation assays at this point showed that only the S stereoisomer was active—to approximately the same level as induced by PS-oligos with stereorandom phosphorothioate linkages. This loss of activity of the R stereoisomer most likely resulted from rapid degradation of the oligonucleotides rather than from reduced interaction with the CpG receptor because PS-oligos in which only the CpG dinucleotide was stereodefined were most stimulatory when the CpG was Rp but not when the CpG was Sp. These studies demonstrate that the sense of P-chirality of PS-oligos plays a major role in determining the biologic activities of CpG motifs. Rp-chirality at the CpG is preferred for best stimulation at early time points, but Sp-chirality of the PS-oligo appears to improve stability and may provide more durable effects in prolonged tissue culture systems.**

## INTRODUCTION

ASIDE FROM ITS ROLE IN CARRYING the genetic code, DNA has been shown recently to function as a signaling molecule (reviewed in Krieg, 2002). The immune systems of higher eukaryotes appear to have evolved a mechanism to detect prokaryotic nucleic acids based on their content of unmethylated CpG dinucleotides in particular base contexts (Krieg et al., 1995). Unmethylated CpG dinucleotides are common in bacterial DNA, but are

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underrepresented (CpG suppression) and are methylated in vertebrate DNA (Bird, 1987). Phosphorothioate oligodeoxynucleotides (PS-oligos) containing these unmethylated CpG dinucleotides in immune stimulatory base contexts (CpG motifs) trigger humoral immunity by inducing B cell activation, proliferation, and interleukin-6 (IL-6) and IgM secretion (Krieg et al., 1995; Yi et al., 1996; Klinman et al., 1996). Such CpG PS-oligos also directly activate monocytes and macrophages to secrete Th1-like cytokines (Ballas et al., 1996; Cowdery et al., 1996; Halpern et al., 1996). This leads to the activation of natural killer (NK) cell lytic activity and interferon-γ (IFN-γ) secretion (Ballas et al., 1996; Cowdery et al., 1996; Chace et al., 1997). CpG motifs are now known to be recognized by a specific receptor, toll-like receptor-9 (TLR-9) (Hemmi et al., 2000), that is expressed in certain subsets of immune cells, such as B cells and particular dendritic cells (reviewed in Krieg, 2002). The interaction of TLR-9 with CpG motifs in these cells cause rapid activation of cell signaling pathways, including mitogen-activated protein kinases (MAPK), such as c-Jun N-terminal kinase (JNK), and nuclear factor-κB (NF-κB).

Native DNA (PO-DNA) is rapidly degraded by a large number of exonucleases and endonucleases in living cells. Therefore, it may not be surprising that CpG motifs in a nuclease-resistant PS backbone have markedly enhanced immune stimulatory effects compared with the same sequence with a PO backbone (Krieg et al., 1995, 1996; Zhao et al., 1996b). Normal PO-DNA has no P-chiral centers, but the PS modification replaces one of the two nonbridging oxygens on the internucleotide linkage with a sulfur atom, which creates a new chiral center. Thus, each internucleotide PS linkage can be either of Rp or Sp absolute configuration (Stec and Wilk, 1994).

As native DNA is P-prochiral, it is not evident whether the proteins that interact with DNA would do so in the case of their P-chiral congeners in a P-stereoselective manner. For example, DNA-dependent RNA polymerases accept as substrates nucleoside 5′-α-thiotriphosphates (NTPαs) of Sp configuration and produce PS-oligos of Rp configuration at each internucleotide PS linkage (Burgers and Eckstein, 1978; Griffiths et al., 1987). Several studies have been performed using stereoenriched or stereopure PS-oligos to investigate the question of stereospecificity of interactions of PS-oligos with proteins. Some interactions with DNA are clearly specific for the Rp stereoisomer. For example, the degradation of DNA by snake venom phosphodiesterase or human 3′- exonuclease is Rp selective and is extremely inefficient with PS-DNA substrates of Sp configuration (Burgers et al., 1979; Tang et al., 1995; Koziolkiewicz et al., 1997; Gilar et al., 1998). As a result, [All-Sp-PS]-DNA is intracellularly far more stable than its [All-Rp-PS] counterpart. Likewise, the RNase H cleavage of RNA/DNA duplexes is faster for the Rp stereoisomer of the DNA component (Koziolkiewicz et al., 1995). The avidity of PS-oligos to complementary RNA has been reported to be higher for the Rp stereoisomer than for the Sp stereoisomer (Tang et al., 1995; Koziolkiewicz et al., 1995; Yu et al., 2000). On the other hand, some experimental systems are reported to recognize only [Sp-PS]-oligos. The nuclease P1 preferentially binds and cleaves [Sp-PS]-oligos over Rp (Potter et al., 1983). Otherwise, EcoRI and *Serratia marcescens* endonucleases cleave PS scissile bonds in an Rp-stereoselective manner (Connolly et al., 1984; Koziolkiewicz et al., 2001) that is influenced by the stereochemistry of PS modifications of neighboring internucleotide linkages (Lesser et al., 1992; Kurpiewski et al., 1996).

Certain sequences of DNA are able to form four-stranded structures, so called *i*-motifs, that reportedly are more stable with the Sp stereoisomer (Kanehara et al., 1997), whereas G-tetraplexes formed by Rp-oligos are more stable than those formed by isosequential Sp stereoisomers (Hawley et al., 1999). Structural features influence antisense activity of PS-oligos. PS-oligos interacting with complementary target RNA via Watson-Crick hydrogen bonding have appeared to be more active as All-Sp stereomers (Stec et al., 1997), whereas those able to form higher structures due to the presence of G-quartet motifs have been reported to have greater antisense activity in certain cells and *in vivo* experimental systems as All-Rp isomers (Fearon et al., 1997).

In contrast to these stereoselective biologic activities, the interaction of nucleic acids with creatine kinase can be selective for either the Rp or the Sp stereoisomer depending on the cation present (Burgers and Eckstein, 1980). The binding of PS-oligos to basic fibroblast growth factor (bFGF), CD4, laminin, and fibronectin is not stereoselective (Benimetskaya et al., 1995). Thus, the interactions of nucleic acids with proteins can be selective for the Rp stereoisomer and the Sp stereoisomer or can be stereoindependent.

To our knowledge, only a single study has examined the effect of P-chirality on the immune stimulatory effects of CpG oligos. Yu et al. (2000) compared stereoenriched (not stereopure) PS-oligos for their ability to induce spleen cell proliferation. In that study, a 19-mer sequence containing a single CpG motif was found to induce high levels of mouse spleen cell proliferation if the oligo was synthesized with random P-chirality or was enriched for Sp internucleotide linkages, but the proliferation was markedly reduced if the oligo was enriched for Rp internucleotide linkages (Yu et al., 2000). However, that study did not examine the specific role of P-chirality at the CpG dinucleotide, nor did it determine if the Rp CpG oligos would have activity in short-term stimulation assays. The present studies were performed to address these questions.

## MATERIALS AND METHODS

### *Cell culture conditions and reagents*

For B cell proliferation assays, spleen cells from BALB/c mice (4–18 weeks old) were cultured at 2–5 × $10^5$–$10^6$ cells/ml in RPMI for 44 hours in 96-well microtiter plates and then pulsed with 1 $\mu$Ci of $^3$H-thymidine for 4–6 hours before being harvested and having cpm determined by scintillation counting, as previously described (Krieg et al., 1995). For Western blots, WEHI-231 cells (American Type Culture Collection, Rockville, MD) were cultured at 37°C in a 5% $CO_2$ humidified incubator and maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies), 1.5 mM L-glutamine, 50 $\mu$M 2-ME, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin.

### *Oligonucleotides*

Oligodeoxynucleotides (PO-oligos) and stereorandom oligo (deoxynucleoside phosphorothioate)s ([Mix-PS]-oligos) were purchased from Operon Technologies (Alameda, CA) or prepared by the standard phosphoramidite method (Caruthers, 1985; Stec et al., 1984). Oligo sequences and modifications are as shown in Table 1. The oligonucleotide [Mix-PS]-d (TCCATGACGTTCCTGACGTT) ([Mix-PS]-1826) was used as a positive control, as it had been found previously to have strong immune stimulatory effects on mouse cells (Yi et al., 1996). For a CpG PS-oligo with a minimal stimulatory motif, the sequence PS-d (TCAACGTT) was chosen for study as a typical CpG motif with broad immune stimulatory effects representative of the broad family of CpG DNA. This sequence was called [Mix-PS]-2066 when made with a stereorandom backbone. When this octamer sequence was made with a complete or partially stereodefined backbone, the PS-oligo was referred to as either [All-Rp-PS]-2066 or [All-Sp-PS]-2066 when the entire backbone was stereodefined or as [CG-Rp-PS]-2066 or [CG-Sp-PS]-2066 when only the CpG dinucleotide was stereodefined. Other PS-oligos used included CpG PS-d (TCAACGTTGA) ([Mix-PS]-2239) and its All-Rp and All-Sp stereodefined counterparts and the control non-CpG PS-d (TCAAGCTTGA) [Mix-PS]-2240.

Stereodefined PS-oligos were prepared by the oxathiaphospholane method as described (Stec et al., 1995, 1998). The syntheses were performed manually. The first nucleoside units from the 3′-end were anchored to the solid support by a DBU-resistant sarcosinyl linker (Brown et al., 1989). Appropriately protected deoxynucleosidyl monomers possessing 3′-*O*-(2-thio-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane) moiety were synthesized and separated chromatographically into pure P-diastereomers. For synthesis of [CG-Rp-PS]-2066 and [CG-Sp-PS]-2066, unresolved mixtures of both P-diastereomers (in Rp/Sp ratio ca. 1:1) (Stec et al., 1998) were used for assembling of internucleotide linkages of random configuration of P-atoms. All synthesized oligomers were purified by two-step RP-HPLC: DMT-on (retention times 23–24 minutes) and DMT-off (retention times 14–16 minutes). The chromatographic system was an ODS Hypersil column, 5 $\mu$m, 240 × 4.6 mm, 0–40% $CH_3CN$ in 0.1 M triethylammonium bicarbonate, pH 7.5, gradient 1%/min. Their purity was assessed by polyacrylamide gel electrophoresis.

TABLE 1. OLIGONUCLEOTIDES USED

| *Name* | *Sequence and chirality* |
|---|---|
| 1826 | T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*C*G*T*T |
| 2066 | T*C*A*A*C*G*T*T |
| Rp (2066) | T$^R$C$^R$A$^R$A$^R$C$^R$G$^R$T$^R$T |
| CG Rp (2066) | T*C*A*A*C$^R$G*T*T |
| F-Rp (2066) | F-T$^R$C$^R$A$^R$A$^R$C$^R$G$^R$T$^R$T |
| Sp (2066) | T$^S$C$^S$A$^S$A$^S$C$^S$G$^S$T$^S$T |
| CG Sp (2066) | T*C*A*A*C$^S$G*T*T |
| F-Sp (2066) | F-T$^S$C$^S$A$^S$A$^S$C$^S$G$^S$T$^S$T |
| 2239 | T*C*A*A*C*G*T*T*G*A |
| Rp (2239) | T$^R$C$^R$A$^R$A$^R$C$^R$G$^R$T$^R$T$^R$G$^R$A |
| Sp (2239) | T$^S$C$^S$A$^S$A$^S$C$^S$G$^S$T$^S$T$^S$G$^S$A |
| 2240 | T*C*A*A*G*C*T*T*G*A |

*Indicates stereorandom PS internucleotide linkage.

$^R$Indicates Rp PS internucleotide linkage.

$^S$Indicates Sp PS internucleotide linkage.

F, 5′-fluorescein.

For studies of PS-oligo uptake, fluorescein-conjugated stereoregular PS-oligos with the 2066 sequence were prepared by solid-phase elongation of manually synthesized stereodefined PS-oligomers. After the detritylation step, fluorescein phosphoramidite (ChemGenes Corporation, Ashland, MA) (working concentration 125 mg/ml) and 1-*H*-tetrazole were routinely added (coupling time 120 seconds), followed by sulfurization with S-Tetra reagent (Stec et al., 1993). Cleavage from the support and deprotection were performed with concentrated ammonium hydroxide for 1 hour at ambient temperature and 4 hours at 55°C, respectively. The resulting oligomers were purified by one step RP-HPLC. Because of remarkable hydrophobicity of fluorescein moiety, the Rp and Sp oligomer was eluted at retention times 14.5, 14.8, and 14.7, and 15.0 minutes, respectively, that is, at the end of failed sequences. In both cases, two p-diastereomers were eluted due to nonstereospecificity of the phosphoramidite/sulfurization method of elongation with the fluorescein monomer.

### *Western blot analysis*

Cells were harvested and resuspended in fresh medium at a concentration of $2 \times 10^6$ cells/ml. Cells then were allowed to rest for 4 hours prior to a 40-minute stimulation. Cells were harvested and washed three times with cold phosphate-buffered saline (PBS), and lysed in 0.05 M Tris, pH 7.4, 0.14 M NaCl, 1% NP-40, 0.001 M $Na_3VO_4$, 0.01 M NaF, 4.3 mg/ml $\beta$-glycerophosphate, 0.002 M DTT, 50 $\mu$g/ml PMSF, 12.5 $\mu$g/ml antipain, 12.5 $\mu$g/ml aprotinin, 12.5 $\mu$g/ml leupeptin, 1.25 $\mu$g/ml pepstatin, 19 $\mu$g/ml bestatin, 10 $\mu$g/ml phosphoramidon, 12.5 $\mu$g/ml trypsin inhibitor by freezing and thawing followed by a 30-minute incubation on ice. The samples were centrifuged at 10,000*g* for 10 minutes at 4°C. The supernatants were saved as whole cell lysates for further analysis. Equal amounts of whole cell lysates (20 $\mu$g) were boiled in SDS sample buffer for 5 minutes before being subjected to electrophoresis on an 11% denaturing polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes using a semidry blotter (Bio-Rad Laboratories, Hercules, CA). Blots were blocked with 5% nonfat milk before hybridization with phospho-SAPK/JNK (Cell Signaling Technology, Beverly, MA), IκB-$\alpha$, and JNK1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Blots were visualized using enhanced chemiluminescence reagents (ECL) (Amersham International, Piscataway, NJ) according to the manufacturer's protocol.

## RESULTS

### *Induction of spleen cell $^3$H-thymidine incorporation by the Sp stereoisomer of CpG PS-oligos*

In order to determine the stereospecificity of the immune stimulatory effects of CpG DNA, BALB/c spleen cells were cultured with stereodefined octanucleotides PS-d (TCAACGTT; sequences in Table 1) in which all of the internucleotide linkages are either Rp or Sp configuration at the concentrations indicated in Table 2. The cells were cultured for 48 hours, which allows sufficient time for B cells to be induced to proliferate by the CpG motifs (Krieg et al., 1995). The stereorandom [Mix-PS]-2066, possessing a CpG motif, induced strong dose-dependent spleen cell proliferation (Table 2). The Sp isomer also induced proliferation and appeared to be marginally more potent than [Mix-PS]-2066. In contrast, the Rp stereoisomer did not induce any detectable proliferation, which was consistent with the findings of Yu et al. (2000).

TABLE 2. INDUCTION OF SPLEEN CELL PROLIFERATION BY SP STEREOISOMER OF CPG OCTAMERS[a]

| *Oligo* | *Concentration (μM)* | *cpm* | *SI*[b] |
|---|---|---|---|
| None (medium) | | 2,170 | 1 |
| 2066 (stereo-random CpG) | 0.4 | 3,154 | 1.5 |
| 2066 (stereo-random CpG) | 2.4 | 16,525 | 7.6 |
| 2066 (stereo-random CpG) | 4.8 | 30,811 | 14.2 |
| Rp (2066) | 0.4 | 1,207 | 0.6 |
| Rp (2066) | 2.4 | 985 | 0.5 |
| Rp (2066) | 4.8 | 640 | 0.3 |
| Sp (2066) | 0.4 | 9,567 | 4.4 |
| Sp (2066) | 2.4 | 35,372 | 16.3 |
| Sp (2066) | 4.8 | 43,591 | 20.1 |
| Rp (2066) + 2066[c] | 0.4 | 1,597 | 0.7 |
| Rp (2066) + 2066 | 2.4 | 10,255 | 4.7 |
| Rp (2066) + 2066 | 4.8 | 15,841 | 7.3 |

[a]Data shown are from one of three experiments performed, with similar results.

[b]SI, stimulation index compared to medium control.

[c]Each of the two PS-oligos was added to the indicated concentration at the start of culture.

Our previous studies had demonstrated that decamer CpG PS-oligos have improved immune stimulatory effects compared with the octamers used in the first experiments. Therefore, these spleen cell proliferation experiments were repeated using the construct PS-2239, which was synthesized either as a stereorandom [Mix-PS]-2239 or in the All-Rp or All-Sp form. Again, both the [Mix-PS]-2239 and the [All-Sp-PS]-2239 induced strong $^3$H thymidine incorporation in a dose dependent manner (Fig. 1). However, in this case, the [All-Rp-PS]-2239

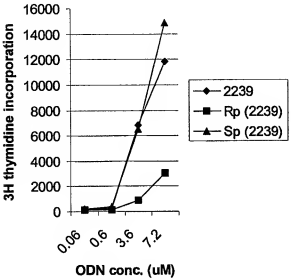


**FIG. 1.** Induction of spleen cell proliferation by decamer CpG PS-oligos. Mouse spleen cells were cultured with the indicated PS-oligo at the concentrations shown for 44 hours, and proliferation assays were performed as described in Materials and Methods. The PS-oligos all had the same sequence, differing only in chirality of the internucleotide linkages, as indicated. The data shown are from one of five experiments performed, with similar results.

was also able to induce a substantial increase in cell proliferation at the highest concentrations, indicating that it retained at least partial stimulatory activity (Fig. 1).

### *Preference for Rp-chirality at the CpG dinucleotide in octamer PS-oligos*

It remained unclear if the apparent preference for the Sp stereoisomer in the initial experiments resulted from an effect within the CG dinucleotide itself or if this effect may be outside the CG. In order to determine this, two octamers PS-2066 were synthesized in which the backbone was stereorandom except for the linkage between the central CG, which was defined as either Sp or Rp. Surprisingly, this experiment appeared to give the opposite result from those using PS-oligos in which the entire backbone was stereoregular, as [CG-Rp-PS]-2066 caused as strong an increase in spleen cell $^3$H thymidine incorporation as the control stereorandom PS-oligo (Fig. 2). In contrast, PS-oligo [CG-Sp-PS]-2066 was essentially inactive (Fig. 2).

### *Inhibition of spleen cell $^3$H thymidine incorporation by the R stereoisomer of CpG PS-oligo*

The level of $^3$H thymidine incorporation in the wells of spleen cells treated with the Rp stereoisomer was lower than in the control wells, suggesting possible inhibitory activity, although no cytotoxicity was apparent on microscopic examination of the cells (data not shown). Indeed, when cells were cultured with an equimolar mixture of the [Mix-PS]-2066 and the All-Rp stereoisomer, there was an approximate 50% reduction in the level of $^3$H-thymidine incorporation compared with cells cultured with only the [Mix-PS]-2066 (Table 2).

### *Preferential immune stimulation by [Rp-PS]-oligos at early time points*

The $^3$H-thymidine incorporation assays performed in the preceding experiments are vulnerable to an artifact resulting from PS-oligo degradation, with release of cold thymidine that competes with the labeled material and artificially suppressing its incorporation (Matson and Krieg, 1992). Previous studies have demonstrated that [Rp-PS]-oligos are far more susceptible to nuclease degradation than their Sp counterparts (Yu et al., 2000). Thus, it was possible that the apparent lack of stimulatory effect of the [Rp-PS]-oligo in our $^3$H-thymidine incorporation assays may have been a misleading artifact that did not reflect the true effects of the [Rp-PS]-oligo. In order to detect the stimulatory effects of the [Rp-PS]-oligo at an early point before the PS-oligo can be degraded and as an independent biologic assay for CpG-induced stimulation, we tested the ability of these PS-oligos to induce rapid phosphorylation of the regulatory MAPK, JNK.

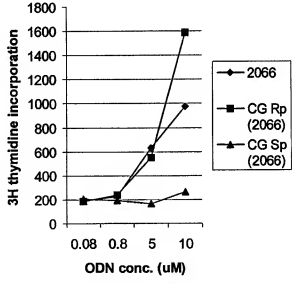


**FIG. 2.** Induction of spleen cell proliferation by CpG stereodefined PS-oligos. Mouse spleen cells were cultured with the indicated PS-oligo at the concentrations shown for 44 hours, and proliferation assays were performed as described in Materials and Methods. The PS-oligos all had the same sequence, differing only in chirality of the internucleotide linkages at the CpG dinucleotide, as indicated. Outside of the CpG dinucleotide, all of the linkages were stereorandom in all three PS-oligos. The data shown are from one of three experiments performed, with similar results.

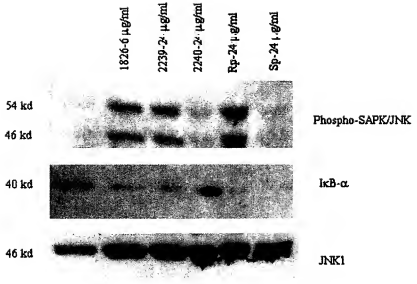


**FIG. 3.** Induction of JNK phosphorylation and IκB degradation by CpG oligos. WEHI-231 cells were treated with the PS-oligo indicated in the figure or with no PS-oligo (Control) and harvested after a 40-minute culture. Cell extract (20 μg) was loaded into each well for PAGE. After transfer, membranes were subjected to Western blotting with antibodies specific for the indicated proteins, as described in Materials and Methods. PS-1826 and PS-2239 are stereorandom positive control PS-oligos, and PS-oligo 2240 is a stereorandom non-CpG PS negative control PS-oligo. Rp and Sp are the stereospecific PS-oligos with the same sequence as CpG PS-oligo 2239. The bottom blot shows that all cell samples contained comparable amounts of total JNK protein. The data shown are from one of three experiments performed, with similar results.

Surprisingly, we found that on treatment of WEHI-231 cells with CpG sequences PS-1826 and PS-2239, within 40 minutes JNK phosphorylation was induced strongly not by the [Sp-PS] isomers but only by the stereorandom [Mix-PS] and by [Rp-PS] isomers (Fig. 3, top). A control non-CpG [Mix-PS]-2240 did not induce detectable JNK phosphorylation. All WEHI-231 cell samples contained comparable amounts of total JNK protein (Fig. 3, bottom).

Although no effect of the CpG [Sp-PS]-oligo could be detected in the JNK phosphorylation assay, the oligo was biologically active in this experiment because the level of the inhibitory protein IκB-α was reduced by all of the CpG PS-oligos, regardless of stereoisomer, but not by the control non-CpG PS-2240 (Fig. 3, middle).

### *Stereo-independence of PS-oligo cell surface binding and uptake*

One potential explanation that could contribute to the observed differences in bioactivity of the PS-oligo stereoisomers is that cell binding or uptake of the PS-oligos may be stereodependent. To test this possibility, P-stereodefined PS-oligos with the 2066 sequence were synthesized with 5′ fluorescent tags and incubated with WEHI-231 cells. Consistent with the results of past studies, the PS-oligos showed a concentration-dependent and temperature-dependent pattern of cell uptake (Table 3). Notably, there was no detectable difference in the binding or uptake of the Rp or Sp PS-oligos in the WEHI-231 cells.

## DISCUSSION

These studies demonstrate that ODN p-chirality can have apparently opposite effects on the immune activity of a CpG ODN, depending on the time at which activity is measured. At an early point of 40 minutes, the Rp but not the Sp stereoisomer of PS CpG ODN induces JNK phosphorylation in mouse B cells. In contrast, when assayed at a late time of 44 hours, the Sp but not the Rp stereoisomer is active in stimulating spleen cell proliferation. We show that this difference in the kinetics and bioactivity of the Rp and Sp stereoisomers does not result from any difference in cell uptake but rather most likely is due to two opposing biologic roles of the p-chirality. First, the enhanced activity of the Rp stereoisomer compared with the Sp for stimulating immune cells at early times indicates that the Rp may be more effective at interacting with the CpG receptor, TLR-9, or inducing the downstream signaling pathways. On the other hand, the faster degradation of the Rp PS-oligos compared with the Sp results in a much shorter duration of signaling, so that the Sp PS-oligos appear to be more biologically active

TABLE 3. BINDING AND UPTAKE OF PS-OLIGOS ARE STEREO-INDEPENDENT[a]

| | *F-Rp (2066) MFI* | *F-Sp (2066) MFI* |
|---|---|---|
| Cell surface binding at 0.25 $\mu$M | 12.1 | 11.0 |
| Cell surface binding at 0.5 $\mu$M | 34.4 | 36.5 |
| Cell uptake at 0.25 $\mu$M | 24.6 | 21.0 |
| Cell uptake at 0.5 $\mu$M | 58.9 | 61.8 |

[a]WEHI-231 cells were cultured with the indicated stereo-specific 2066 PS-oligo containing a 5′ fluorescein at the indicated concentration for either 30 minutes at 4°C (Cell surface binding) or for 2 hours at 37°C (Cell uptake). Cells were washed quickly with chilled FACS buffer and immediately analyzed by flow cytometry for mean fluorescence intensity (MFI) as previously described (Zhao et al., 1996a). The data shown are from one of five experiments performed, with similar results.

when tested at later times. The rapid degradation of Rp PS-oligos in serum or in cells needs to be considered in the design and interpretation of experiments using stereospecific oligos.

Our studies reveal the novel finding that the previously reported relative lack of immune stimulation by Rp PS-oligos is due only to their nuclease lability, not to an inherent inability to stimulate the CpG receptor and downstream pathways. When tested for their ability to stimulate JNK phosphorylation, which indicates activation of this MAPK pathway, the Rp ODN appeared to be the most active, followed by the stereo-random oligo, but with no detectable activity of the Sp ODN (Fig. 3). However, when these ODNs were compared for their ability to activate the NF-$\kappa$B pathway, as measured by degradation of the inhibitory protein I$\kappa$B-$\alpha$, all of the CpG ODN were active, although the non-CpG control failed to induce I$\kappa$B-$\alpha$ degradation. Thus, the Sp ODN is still biologically active. Its failure to induce the JNK pathway could be related to differences in the kinetics of activation of the JNK and NF-$\kappa$B pathways, but because of the limited amounts of the stereospecific ODN that were available for testing, we were unable to confirm this hypothesis.

Our experiments revealed a surprisingly strong effect of the P-chirality at the CpG dinucleotide itself. In comparison to the stereorandom CpG oligo 2066, the congener in which the single CpG dinucleotide was linked in Rp was slightly more active, whereas the congener containing an Sp linkage was nearly inactive for inducing spleen cell proliferation (Fig. 2). The loss of activity of the Sp congener supports our hypothesis that the TLR-9 receptor may not be indifferent to the chirality of the CpG dinucleotide in the DNA with which it interacts but may actually be stimulated better by the Rp stereoisomer. Thus, the stimulatory effect of the stereorandom oligo is probably not only because of the presence of 50% Sp linkages that retard degradation but also because half of the oligo molecules will have Rp chirality at the CpG dinucleotide, which appears to enhance the immune stimulatory effects. It remains unclear whether the chirality of the internucleotide linkages adjoining or distant from the CpG dinucleotide may also modulate the immune activity of a CpG PS-oligo. As native DNA is P-chiral, it is clear that the immune stimulatory effects of CpG oligos must not require a chiral backbone.

The nuclease sensitivity of Rp PS linkages has important implications for interpretation of pharmacokinetic (PK) and metabolism studies of PS-oligos in humans or animals. The predominant serum nuclease activity is known to be a 3′ exonuclease. In a typical stereorandom PS-oligo solution, the last 3′ internucleotide linkage will be expected to be of Rp chirality in one half of the molecules. Therefore, in these 50% of the PS-oligo molecules, the terminal 3′ base will be cleaved fairly rapidly after i.v. infusion. The second from the end 3′ internucleotide linkage should be of Rp chirality in one half of these molecules, and, therefore, in 25% of the starting PS-oligo molecules, the 3′-end may be expected to be shortened by 2 bases relatively rapidly. This *in vivo* base-clipping process involving the 3′ Rp internucleotide linkages may be expected to continue until the 3′ internucleotide linkage is of Sp configuration, after which further cleavage would be delayed. Therefore, if the PS-oligos were synthesized to have an Sp 3′ terminal linkage, they should have much slower degradation and a different PK profile than stereorandom PS-oligos. This should make it possible to use somewhat shorter ODN for *in vivo* applications. In some cases, however, it may be desirable to design oligos for more rapid degradation, in which case one could simply use the Rp linkages at or near the 3′-end or 5′-end, or internally, to allow for more rapid cleavage of the oligo into smaller fragments. Such an approach should reduce the accumulation of oligos in tissues, such

as the liver and kidney, and should reduce or prevent adverse effects of high oligo concentrations on these organs. In designing optimized oligos for antisense applications, the enhanced RNA binding of the Rp stereoisomer points to the desirability of having as much of the internal core of the ODN in Rp configuration as possible. To avoid immune stimulation with antisense oligos containing one or more CpG dinucleotides, one could specify the CG linkage (s) to be of Sp chirality. On the other hand, an optimized CpG ODN for immunostimulatory applications may be one in which all of the internucleotide linkages except the CpG would be of Sp chirality. Although the stereorandom backbone CpG PS-oligos used in current human clinical trials appear to be highly active at enhancing immune responses to vaccines and have been well tolerated (Krieg, 2003), our results point to the potential for designing more effective chimeric oligos. Of course, such chimeric PS-oligos would still be susceptible to cleavage from endonucleases. Further studies will be required to explore these possibilities.

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